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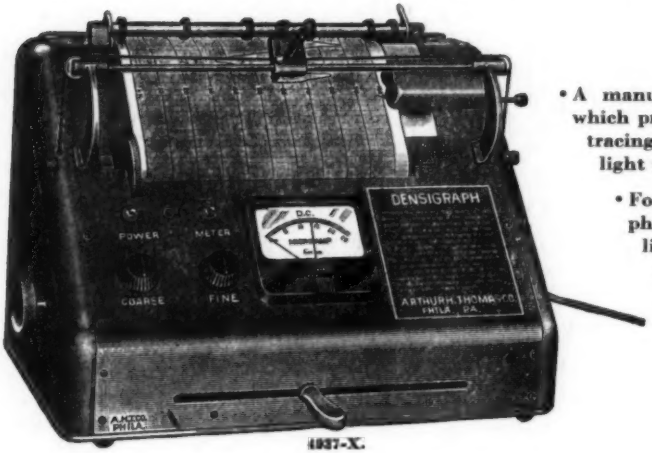

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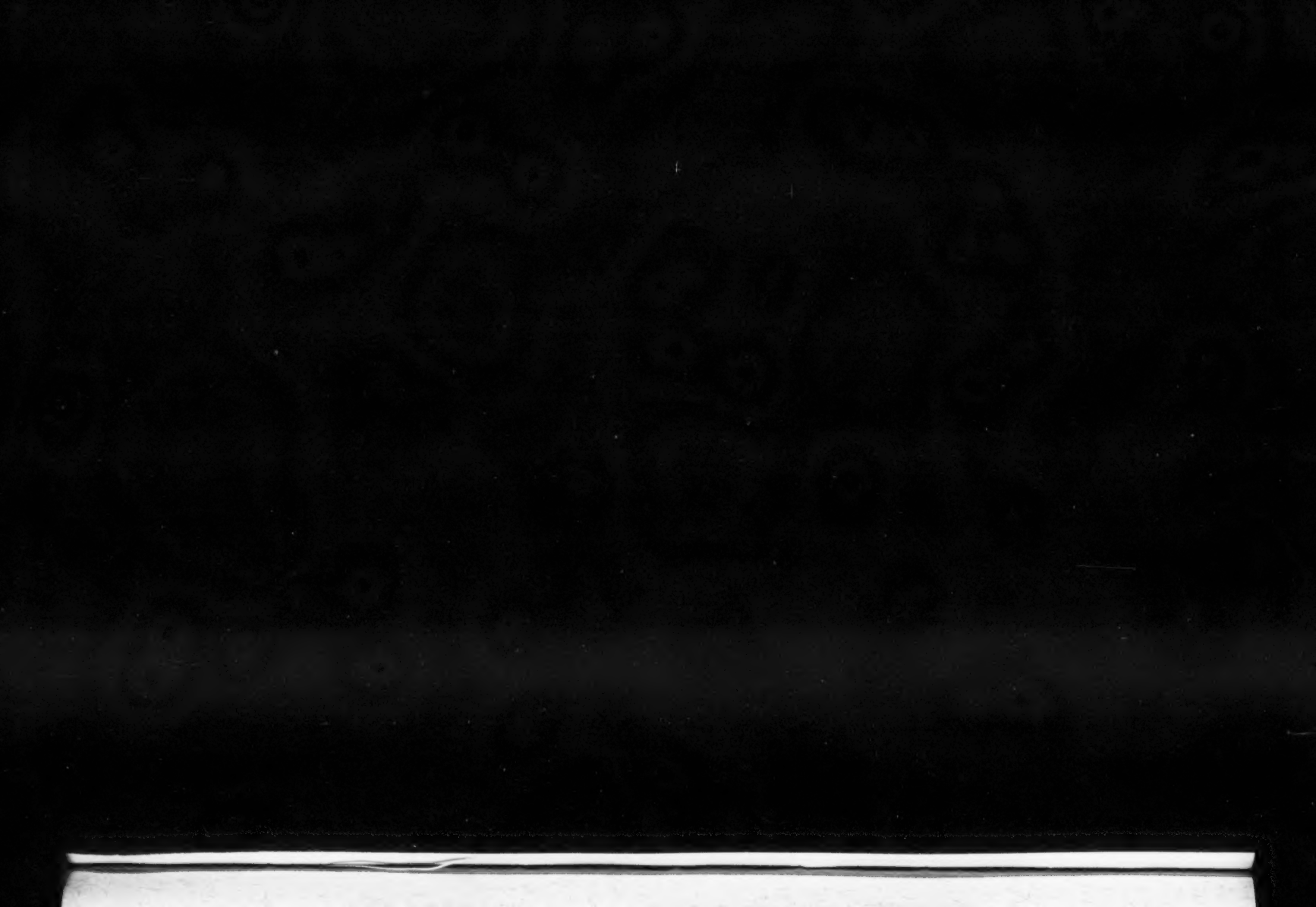

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THE RELATIONSHIP BETWEEN pH AND THE ACTIVITY OF CHOLINESTERASE FROM FLIES

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In order to make useful comparisons of similar enzyme systems from different organisms, or of the response of a given system to different chemical agents, some understanding of the possible effect of changes in the conditions of assay is essential. This requirement becomes especially conspicuous in attempts to correlate results from laboratories whose techniques are not identical, as illustrated, for example, by certain discrepancies that will be discussed below. In commencing a series of studies intended to bring to light the distinctive properties of insect cholinesterases (ChE's), we have therefore found it expedient to examine in detail the effects of altering our experimental conditions, and have already reported the results of tests in which the activity of fly head ChE was measured in relation to the composition of the suspending medium (Chadwick, Lovell and Egner, 1953).

Another factor with significant influence *in vitro* on the rate of hydrolysis of acetylcholine (ACh) by ChE is the hydrogen ion concentration. For most ChE's that have been studied in this connection, enzymic activity was maximal somewhat on the alkaline side of neutrality, fell rather sharply at still higher pH values, and declined more gradually as hydrogen ion concentration was increased. The pertinent references are discussed by Augustinsson (1948); see also Table IV below.

Three studies of the problem with insect material have been reported. Tahmisian (1943) found a relationship of typical form and a pH optimum of 8.5 with the enzyme from developing grasshopper eggs. Stegwee (1951), working with central nervous tissue of the beetle, *Hydrophilus,* and the roach, *Periplaneta,* recorded rather sharp optima at pH 7.4. Data of Babers and Pratt (1950) with fly head suspensions are in contrast with these and all other reports in that they indicate a peak in activity at about pH 5.75. In their preparations, ChE activity was maintained near half peak level between pH 6.25 and 9.00, and decreased abruptly at higher pH values, as at values below 5.50. They comment (p. 61) that "this activity over such a wide pH range not only was unexpected but is also unexplained"; however, the most unusual feature of their results is the position of the optimum.

At the time their work was published, our investigations of the same problem were already in progress, and it was apparent immediately that our observations did not agree fully with theirs. We therefore extended the scope of the experiments, first in order to establish more firmly the nature of the relationship between

pH and the activity of fly head ChE, and secondly with the hope of reconciling the differences between our data and those of Babers and Pratt. In addition, since the reduction in activity at low and high pH was found to involve some irreversible inactivation of the enzyme, we made measurements of this aspect of the process.

Experimental

Culture of flies (*Musca domestica* L.) preparation of head suspensions, and our application of Glick's (1937) titrimetric method of measuring ChE activity have been described in an earlier report (Chadwick, Lovell and Egner, 1953). In the present experiments, data were obtained at 25.0 degrees C. on 20-ml. aliquots, containing the equivalent of 20 heads each, with three suspension media. Of these, the first was buffer: NaCl, 26.30 gm.; KH_2PO_4, 3.85 gm.; NaOH, 1.00 gm.; H_2O, to one liter. This solution was designed to promote maximal enzymic activity, which had been found to require the presence of a salt at about 0.5 N concentration; and was buffered lightly, so as to minimize fluctuation of pH during assay and yet retain sufficient sensitivity for accurate determination of the rate of production of acid. Since our results with this medium differed considerably from those reported by Babers and Pratt (1950), whose suspensions contained glycerol, a second series of observations was made with head tissue ground and assayed in 30 per cent glycerol. In a third set of experiments, the brei was suspended in de-ionized water. With all the suspensions, pH was adjusted to the desired level by addition of NaOH or HCl.

The total acid production during test periods of approximately 15 minutes was corrected by subtraction of the acid produced under conditions that were identical except that the enzyme had been inactivated by exposing the stock brei overnight or for a longer time to 1×10^{-5} *M* diisopropyl fluorophosphate (DFP). The net, or enzymic, activity was then converted into micromoles of ACh.Br hydrolyzed per ml. (*i.e.*, per head) per hour.

The rate of permanent inactivation of fly head ChE at low and high pH was determined on aliquots that were incubated at the desired pH value for definite periods of time, and then readjusted rapidly to pH 8.0 before addition of ACh.Br for assay.

In all these experiments, the concentration of substrate at the beginning of measurement was 0.015 *M*. Other concentrations were used in a few experiments for special purposes, as cited in the discussion.

Results

Average rates of enzymic hydrolysis of 0.015 *M* ACh.Br at various pH values in the three media tested are given in Table I. The data have been plotted in Figure 1 as percentages of the average value determined for these tissue samples in buffer at pH 8.0.

Also shown in Table I are the corresponding corrections for non-enzymic hydrolysis. These were evidently not identical in the several media. As pointed out in our previous paper, such variation results in part from the fact that pH

is not truly constant in our method of measurement. Each addition of NaOH during titration pushes pH to the alkaline side of the chosen value, and, for a given amount of alkali, such excursions are greater the more weakly buffered the solution. Error from this cause is not overly significant at pH 8.0 and below, but increases rapidly in more alkaline solutions, where the rate of non-enzymic hydrolysis of ACh is rising steeply with increase in pH. Since ChE activity, as measured in well buffered solutions, increases but slightly above pH 8.0, the error resulting from fluctuation of pH in the experimental samples is largely in the nonenzymic fraction and should theoretically be compensated by the nearly equal error in the controls. In practice, however, we found it difficult to obtain satisfactorily consistent results at pH 9.5 and 10.0.

In order to supply some indication of the range of variation encountered, we have computed standard errors for the means in each series except for those

TABLE I

Enzymic activity of fly head ChE as a function of pH

pH	4.0	5.0	5.5	6.0	7.0	7.5	8.0	8.5	9.0	9.5	10.0
In buffer											
Net rate*	nil	0.34	1.97**	2.05	4.05	4.39	4.88	5.08	5.27	4.64	1.17
±s.e.	—	0.03	—	0.13	0.18	0.11	0.10	0.24	0.22	0.36	0.83
n***	4	10	5	9	10	11	31	10	13	10	10
Correction	—	—	−0.03	−0.03	−0.07	−0.09	−0.24	−0.56	−1.52	−4.78	−13.60
In de-ionized water											
Net rate*	nil	0.54	1.42	1.66	2.24	2.54	2.15	1.66	2.88	1.46	0.88
±s.e.	—	0.07	0.18	0.24	0.21	0.24	0.12	0.16	0.51	—	—
n***	6	10	10	10	10	10	17	10	10	5	5
Correction	—	—	−0.01	−0.02	−0.04	−0.28	−0.56	−1.21	−3.10	−7.63	−22.17
In glycerol, 30 per cent											
Net rate*	nil	0.44	0.88	1.27	1.42	0.98	1.37	0.88	1.51	0.63	0.15
n***	5	5	5	6	5	5	13	5	5	5	5
Correction	—	—	—	−0.01	−0.02	−0.25	−0.54	−1.14	−2.63	−4.24	−7.25

* Average net rates, standard errors, and corrections in micromoles ACh.Br hydrolyzed per ml. (= per head) per hour.

** pH, 5.75.

*** n, number of tests.

All runs at 25.0 degrees C.; ACh.Br, 0.015 *M*.

where only 5 determinations were made at each pH level. We report these calculations with some hesitation, first because of the relatively small "n," and secondly because the measurements at different pH values in a single series were not wholly independent. For example, aliquots of a stock brei which showed more than average activity at one pH value tended to give higher than average measurements at all pH levels. Thus, the sampling was not truly random, and on this account the standard errors listed in the table should not be relied on for estimates by the t-test of the significance of differences between means.

The second table shows the average activity remaining in samples that had been incubated at the indicated pH values for 30 minutes and then readjusted to pH 8.0 for assay. In Table III are recorded the results of exposing samples to pH 4.0 for different periods of time, up to two hours. Some few additional data pertinent to these experiments are cited in the discussion.

Table VI shows ChE activity as a function of pS at two pH levels, *viz.*, 6.0 and 8.0.

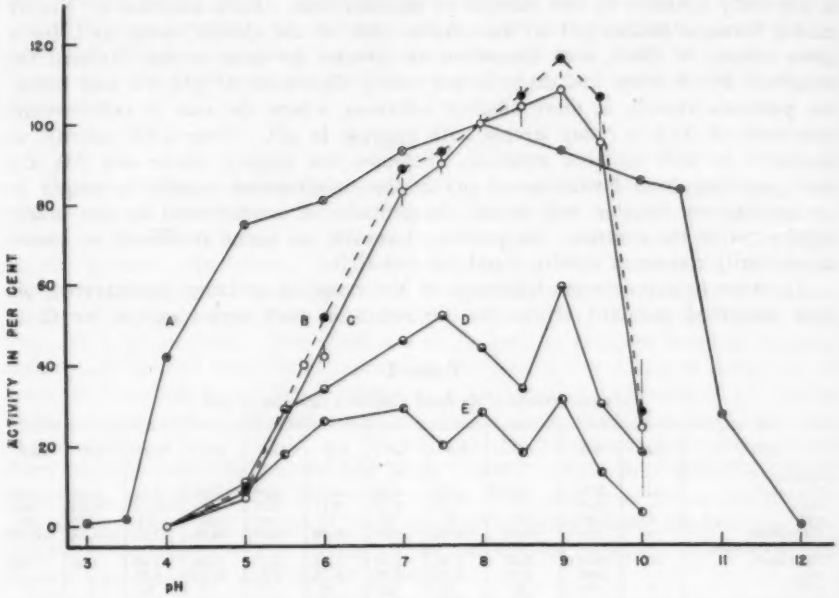

FIGURE 1. Variation in ChE activity of fly heads as a function of pH. Curve A. Residual activity after 30-minute exposures at indicated pH values; all measurements at pH 8.0 in buffer. Curve B. Specific activity at indicated pH values, computed by correcting Curve C for degree of inactivation shown in Curve A. For fuller explanation, see text. Curve C. Activity measured during 15-minute exposures at indicated pH values in buffer. The open circles give the mean values, and the vertical bars indicate the limits for ± 3 s.e. Curve D. Activity measured during 15-minute exposures at indicated pH values in water. Curve E. Activity measured during 15-minute exposures at indicated pH values in 30 per cent glycerol. All data shown have been corrected for non-enzymic hydrolysis.

TABLE II

ChE activity of fly head suspensions after 30 minutes exposure at various pH values

pH	Average enzymic activity (per cent)	Number of observations	pH	Average enzymic activity (per cent)	Number of observations
3.0	1.0	1	8.0	100.0	10
3.5	1.9	5	9.0	93.2	5
4.0	41.9	4	10.0	85.6	5
5.0	75.0	4	10.5	83.2	5
6.0	81.1	5	11.0	27.2	5
7.0	93.4	5	12.0	nil	2

All runs at 25.0 degrees C.; ACh.Br, 0.015 *M*; samples readjusted to pH 8.0 for measurement.

TABLE III

Rate of inactivation of fly head ChE at pH 4.0 as a function of duration of exposure

Time exposed minutes.....	0	15	30	60	90	120
Activity in per cent	100	65.9	41.9	30.1	24.9	20.5
Number of observations	20	5	4	4	4	4

All runs at 25.0 degrees C.; ACh.Br, 0.015 *M*; samples readjusted to pH 8.0 for measurement

DISCUSSION

1. *ChE activity as a function of pH*

Examination of the measurements in buffer convinces us that the ChE of our fly heads differs little, in respect to the effect of pH on activity, from most other ChE's hitherto studied (*cf.* Table IV). The optimum is clearly on the alkaline side, being at least as high as 8.0 and probably as high as 9.0.

In aqueous suspensions or in 30 per cent glycerol, activity was generally low in comparison with observations at corresponding pH values in buffer, with the rates in glycerol somewhat less than those in water. These data provide a further demonstration of the activating effect of 0.5 N salt and the depressant effect of glycerol, to which we called attention earlier (1953). In water or glycerol there appeared to be little significant change in ChE activity over the pH range from

TABLE IV

pH optima of ChE's from various sources

Source of enzyme	pH optimum	Authority
Eggs, developing, *Melanoplus*	8.5	Tahmisian, 1943
Erythrocytes, human	7.6 or above	Plattner *et al.*, 1928
Erythrocytes, human	7.5 to 8.0	Alles and Hawes, 1940
Serum, human	8.2 or above	Plattner *et al.*, 1928
Serum, human	8.0 or above	Easson and Stedman, 1936
Serum, human	8.4 to 8.5	Glick, 1937
Serum, human	8.0 to 8.5	Werle and Uebelmann, 1938
Serum, horse	ca. 8.5	Glick, 1938
Serum, horse	7.2 or above	Kahane and Levy, 1936
Serum, horse	8.0 to 8.5	Werle and Uebelmann, 1938
Heart extract, frog	7.5 or above	Loewi and Navratil, 1926
Electric organ, *Electrophorus*	ca. 8.5	Wilson and Bergmann, 1950
Gastric mucosa, pig	ca. 8.5	Glick, 1938
Brain, rat (also rabbit, guinea pig, cat, dog)	ca. 8.4	Bernheim and Bernheim, 1936
Brain, cat	ca. 8.5	Glick, 1938
c.n.s., *Periplaneta*, *Hydrophilus*	7.4	Stegwee, 1951
Heads, *Musca*	5.75	Babers and Pratt, 1950
Heads, *Musca*	8.0 or above	This paper

6.0 to 9.0, in agreement with the findings of Babers and Pratt (1950); however, variation in our measurements was considerable and the curves are quite irregular.

Neither in these media nor in buffer could we find any evidence for an activity peak in the neighborhood of pH 5.75, as reported by Babers and Pratt. This led us to attempt one final comparison, in which activity at pH 5.75 and 7.0 was measured under conditions as nearly like theirs as we could make them. For this purpose, tissue was prepared in 30 per cent glycerol and diluted 1:6 for assay, which was carried out on 9.0-ml. aliquots that contained 150 mg. of tissue and 0.045 *M* ACh.Br. Babers and Pratt had used 3.0-ml. samples containing 50 mg.

TABLE V

Comparison of ChE activity of fly head suspensions in 5 per cent glycerol at pH 7.0 and 5.75

	pH 7.0	pH 5.75
	ml. 0.02 N NaOH per 3 ml. per 20 minutes	
	1.00	1.05
	1.05	1.03
	0.98	1.09
	1.03	1.02
	1.01	1.01
Average	1.01	1.04
*Correction	−0.04	−0.01
Net	0.97	1.03

* Correction values from Babers and Pratt (1950). All runs at 25.0 degrees C.; ACh.Br, 0.045 *M*; tissue, 150 mg.; total volume, 9.0 ml. Data computed to 3.0 ml. volume for sake of comparison with results of Babers and Pratt.

of tissue, but this volume was too small for our electrodes. As in their tests, acid produced was titrated with 0.02 N NaOH over a 20-minute test period. Five replications were made. The results, corrected for non-enzymic hydrolysis with values taken from Babers and Pratt (1950), are shown in Table V.

These data suggest the following comments. First, activity was nearly equal at both pH values; *i.e.*, evidence for a pronounced peak at pH 5.75 was not forthcoming. Secondly, as was to have been expected, activity per unit weight of tissue, or per head, was intermediate at pH 7.0 between the values previously found with suspensions in water and in 30 per cent glycerol, respectively. Finally we may

TABLE VI

Activity of fly head ChE as a function of substrate concentration at two pH levels

Molar concentration of ACh.Br	0.001	0.003	0.01	0.03	0.10
	Average enzymic activity in micromoles per head per hour				
pH 8.0	4.83	5.11	5.29	4.51	2.83
pH 6.0	1.22	1.77	2.37	2.44	1.71

Each datum is the mean of 5 determinations. All runs on aliquots of the same stock brei in buffer at 25.0 degrees C.

note that the activity of our preparation, at both pH levels, was more than twice the peak value reported by Babers and Pratt (1950). Unless some undetected difference in our methods of preparing the tissue can be held responsible, this observation indicates a possible strain difference between their flies and ours; and should strain differences of this magnitude exist, they could conceivably extend to a shift in the pH optimum from above 8.0 to 5.75. This, however, seems very unlikely in view of the bulk of evidence (Table IV) in favor of an alkaline pH optimum for ChE's in general. The remaining alternative is to ascribe the observation of Babers and Pratt to fortuitous variation in the activity of different breis; *i.e.*, to a somewhat unlikely coincidence of sampling errors, that led them repeatedly to exceptionally high values at pH 5.75. This solution does not appeal to us, since it is obviously indemonstrable and because the same sort of inference could, with equal justification, be applied to our own data; but all our efforts to find a more satisfactory explanation have failed.

Theoretical reasons for anticipating an increase in optimal concentration of substrate as conditions of measurement depart from the pH optimum have been put forward by Wilson and Bergmann (1950). The data in Table VI bear on this question, and do in fact indicate a slight shift of $pS_{opt.}$ in the predicted direction at pH 6.0 as compared with pH 8.0. Although it is of interest that this shift should appear in our results, the presence of the effect will hardly demand correction of the pH-activity data in Table I and Figure 1, for the following reasons. The magnitude of the shift is small, the optima are relatively flat, and the standard concentration of 0.015 *M* ACh.Br used routinely in our experiments is already somewhat above the optimum for pH 8.0.

2. *Inactivation of fly head ChE*

The reduction in activity of fly head ChE at hydrogen ion concentrations that depart appreciably from pH 8.0 is not wholly reversible. This fact raises a question as to what portion of the activity change observed at different pH levels is due to an effect of pH on reaction rate, and what portion to permanent destruction of a fraction of the enzyme. Obviously, data such as those in Table I must reflect a summation of both these processes.

We have attempted in a preliminary manner to separate the two effects by measuring the irreversible inactivation of ChE that results when the suspensions are exposed in buffer to different pH values for a constant period of time. The interval chosen was 30 minutes, this being somewhat longer than the average total exposure during our routine 15-minute determinations. As indicated in Table II, the percentage inactivation observed under these conditions remained within moderate limits until one passed below pH 5.0 or above pH 10.5. Other observations not given in the table showed that, within this pH range, there was little if any additional loss of activity during exposures of as much as two hours; and further that subsequent incubation of the samples at pH 8.0 for as long as 18 hours caused no reversal of the loss that had already occurred.

That inactivation did not take place instantaneously was demonstrated by a series of tests at pH 4.0, where suspensions were held for periods varying from 15 minutes to two hours, before return to pH 8.0 for assay (Table III). Here

the process of inactivation was rapid for the first 30 minutes, and followed a slower course thereafter. Both segments of the relationship have the characteristics of a first order reaction, as indicated in Figure 2.

Below pH 4.0 and above pH 11.0, inactivation was rapid and extensive. As a matter of fact, the enzyme, together with large amounts of eye pigment, was precipitated from aqueous suspensions of head tissue at about pH 5.0 to 5.1. Some 75 per cent of the original activity could be recovered if this precipitate

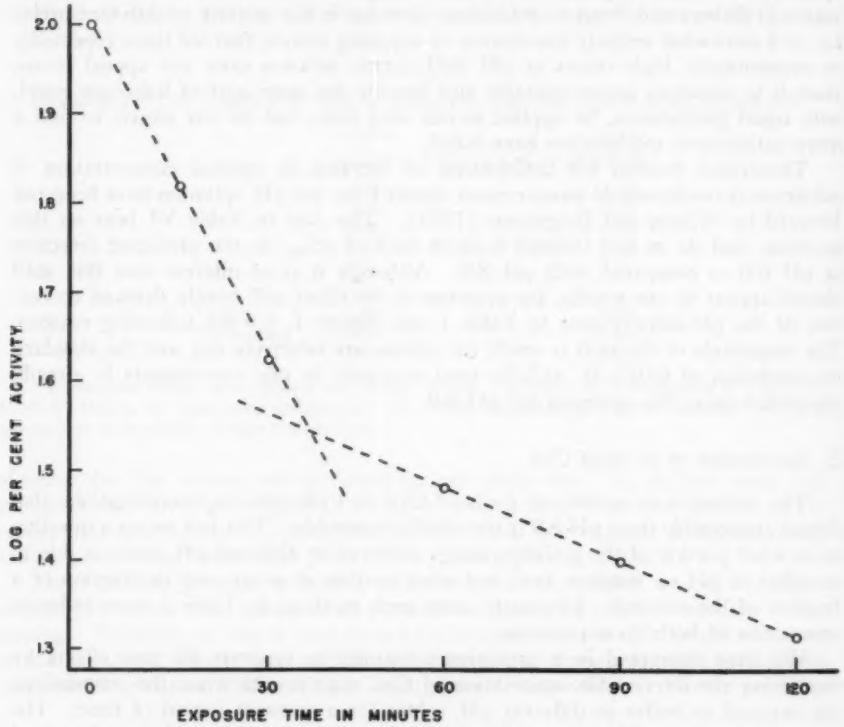

FIGURE 2. Rate of inactivation of fly head ChE in buffer as a function of duration of exposure at pH 4.0. All measurements made at pH 8.0. For further details, see text and Table III.

was quickly re-suspended in buffer at pH 8.0; but it was evident that the enzyme had been altered, since it was no longer as soluble as before precipitation. It was now easily re-separated by light centrifugation. According to Augustinsson (1948), the isoelectric point of several other ChE's has been reported as about pH 4.5. He also notes that precipitation at this level results in more or less permanent solubility changes.

By combining the data of Table II, which show the fraction of enzyme still active after half-hour exposures at the various pH values, with those of Table I,

which give the rates at which similar aliquots were able to hydrolyze substrate while at the same pH levels, it is possible to construct a corrected curve that compensates for changes in the relative amount of active enzyme. Such a curve is shown as B in Figure 1. The divergence from Curve C, which embodies the uncompensated activity data, is slight. This is because 30-minute exposures at pH values between 5.0 and 10.5 inactivated only small fractions of the enzyme, while the effect of pH on reaction rate was already considerable, well within these limits. The compensated curve (B) emphasizes the activity peak at pH 9.0, since the proportion of active enzyme has already begun to fall in this region, whereas the measured activity per unit of tissue has increased slightly above the value determined at pH 8.0. The results also show incidentally that the drop in activity at still higher pH levels is not due wholly to denaturation of the enzyme, since the measured activity decreased more rapidly than the enzyme was destroyed.

These data suggest a pH of about 8.0 as a suitable compromise for experiments where an approach to maximal activity of fly head ChE is desired. Although the true optimum probably lies as much as a full pH unit to the right of pH 8.0, the increase in enzymic activity over this range is slight, whereas the correction for non-enzymic hydrolysis is rapidly becoming larger. At pH 8.0, this correction is less than 5 per cent of the average total activity measured under our conditions; *i.e.*, with tissue concentration at one head per ml.; temperature, 25.0 degrees C.; substrate, 0.015*M*; and salt present in the buffered suspension at about 0.5 N concentration. The correction could be still further reduced by shifting to even lower pH levels, but only with increasing sacrifice of enzymic activity.

Summary

1. Variation in activity of fly head cholinesterase (ChE) was measured titrimetrically at 25.0 degrees C. with ACh.Br 0.015 *M* as substrate, as a function of the pH of the assay medium over the range from pH 4.0 to 10.0. Ground tissue obtained from *Musca domestica* L. was suspended at a concentration of one head per ml. in three media: (1) buffer of composition NaCl, 26.30 gm.; KH_2PO_4, 3.85 gm.; NaOH, 1.00 gm.; H_2O, to one liter; (2) 30 per cent glycerol; (3) de-ionized water.

2. Enzymic activity was greater in buffer than in the other media. The pH optimum was definitely on the alkaline side, being at least as high as pH 8.0 and probably as high as 9.0. In glycerol or water suspensions, enzymic activity changed little between pH 6.0 and 9.0.

3. Some permanent inactivation of the enzyme was observed in half-hour exposures at high and low pH values. This effect was measured over the pH range from 3.0 to 12.0. Between pH 5.0 and 10.5, the degree of inactivation was moderate and essentially complete within 30 minutes. The time course of the process was followed at pH 4.0 for intervals from 15 minutes to two hours, and appeared to involve a rapid phase during the initial 30 minutes and a slower phase thereafter. Both phases had the characteristics of a first order reaction. Inactivation of ChE resulting from exposure to low or high pH was not reversed during subsequent incubation of the sample at pH 8.0 for as long as 18 hours.

4. Correction of the pH-activity curve to allow for changes in the relative amounts of enzyme that result from permanent inactivation requires only minor

alterations, since the effect of pH on reaction rate makes itself felt within pH limits where the degree of permanent inactivation is slight.

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THE AMINO ACID REQUIREMENTS OF THE CONFUSED FLOUR BEETLE, TRIBOLIUM CONFUSUM, DUVAL.

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The rapidly accumulating literature on the nutrition of insects contains comparatively few data on amino acid requirements. However, all present evidence seems to indicate that insects require the ten amino acids which are essential for the rat.

Work on the nutrition of *Tribolium confusum* in a chemically well-defined medium, consisting of casein, glucose or starch, cholesterol, a salt mixture and eight to 10 vitamins of the B-complex, has been previously published by several authors (Fraenkel and Blewett, 1943, 1947; Fraenkel and Stern, 1951; Offhaus, 1952). An entirely successful "synthetic" diet for *Tribolium*, on which growth is as good as on the best natural diets, has not yet been reported. It has only very recently been found that carnitine is required for adult development (French and Fraenkel, 1954). The addition of 1% brewers yeast to a synthetic diet invariably leads to an improvement of growth. However, even in the absence of yeast, *Tribolium* grows sufficiently well to determine the effect of amino acid deficiencies. The results of the present study largely confirm and extend work on similar lines by Lemonde and Bernard (1951).

Methods

The basic diets used in this investigation were derived from diets which had been previously used in work with *Tribolium*. However, the fact that amino acid mixtures were used in the place of casein necessitated certain modifications in the diet. It was desirable to reduce the proportion of amino acids to a relatively low level which would still allow for adequate growth. *Tribolium* grows well on a wide range of carbohydrates ranging in concentration from 5 to 80% of the diet. In most of our previous work the protein level used was 50%. In the present study this was reduced to a total of 15% casein or mixtures of amino acids. In almost all our previous work the carbohydrate in the diets had been glucose. However, in the present study corn starch was used as the carbohydrate in all tests. Glucose could not be used because of the Maillard reaction between sugars and amino acids described by Friedman and Kline (1950a, 1950b). All the starch used in the experiments to be described was from the same batch.

The diets consisted of 15 parts casein or amino acid mixture, 85 parts corn starch, one part cholesterol, 2 parts McCollum's salt mixture no. 185 and the following vitamins of the B-complex (expressed as μg. per gram of the dry diet): thiamin 25, riboflavin 12.5, nicotinic acid 50, pyridoxin 12.5, pantothenic acid 25, choline chloride 500, inositol 250, pteroylglutamic acid 2.5 and biotin 0.25. All the ingredients, except the vitamins, were mixed in the dry state. To ensure a good distribution of those ingredients which were present in very small amounts,

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the mixture of amino acids was first ground with an equal amount of starch, the cholesterol and salts were then added and ground together, and the balance of starch finally added and mixed in. The diets also contained $NaHCO_3$ in amounts of approximately ten per cent of the amino acid mixture (to neutralize free acids). The vitamins were then added in solution in the amounts stated above to add 10% water to the dry diet. After mixing the vitamin solution into the diet with a spatula, the diets were left standing for two days in a constant temperature chamber at about 30° C. and 60–70% relative humidity, and then ground by hand in a mortar.

The tests were performed in shell vials, 1 × 2 inches, with one gm. of dry diet per vial, two vials to each diet. Ten first stage larvae were placed in each vial. All the tests were performed in a constant temperature chamber at 29–30° C. and 60–70 per cent relative humidity.

To assess the efficiency of a diet, two criteria were used. The number of surviving larvae and their average weight were determined after a period long enough to allow larvae on the optimal diet in a particular experiment to reach their maximum weight before pupation had started. This period varied somewhat, according to the composition of the diets, but was usually 20 days. On optimal diets, with glucose as the carbohydrate and the addition of yeast, pupation may occur after 15 days; however, since all the diets contained starch and few contained yeast, the period required for full growth was longer. The date of pupation was then recorded for each individual larva. From these results the average time to pupation was calculated for each test. In some cases the pupae were kept until the adult beetles emerged and the newly-formed beetles were examined. In following this procedure it was considered possible that certain amino acid deficiencies might affect larval mortality, growth rate, pupation or emergence in a different way than others. The most significant data were usually derived from the weights of the larvae. Since slow growth always leads to a delay in the onset of pupation, a positive correlation should exist between weights, growth rate and days to pupation. However, in some experiments, the number of pupae was unexpectedly small. The data concerning adult emergence finally proved to be without significance, since after most of the work was completed, it was discovered that the adults of *Tribolium*, which were grown on artificial diets, were not viable or failed to emerge, unless carnitine was added to the larval diets. There was, however, no indication that carnitine was necessary for larval growth and successful pupation (French and Fraenkel, 1954).

Growth and survival of *Tribolium* vary somewhat in diets run at different times. This may be due to slight changes in temperature and humidity, a difference in the viability of different batches of larvae and possibly other factors which are not too well understood. It makes it necessary to include in each experiment the appropriate positive and negative controls, and to make strict comparisons only between diets run at the same time.

Experiments

A. The amino acids mixtures used, and their effect on three species of insects

In the absence of data about the amino acid requirements of *Tribolium* when this study was initiated, it was considered advisable to start the work with mixtures

which had proved successful with higher animals. Three were used altogether, two of which were amino acid mixtures used by Rose, Oesterling and Womack (1948) with the white rat. The third was one devised by Almquist and Grau (1944) for chicks. Table I gives the percentage composition of these amino acid mixtures. They were at first tested for their effect on the larvae of three beetles, *Tribolium confusum, Tenebrio molitor* and *Dermestes vulpinus*. The diets for *Tribolium* and *Tenebrio* were identical, except for the addition of 1.5 μg. carnitine per gram of the diet for *Tenebrio*. *Tenebrio* and *Tribolium* received 15% amino acids whereas *Dermestes*, which is a typical protein feeder, received 30% amino

TABLE I

Composition of the amino acid mixtures used in studies of the amino acid requirements of Tribolium confusum, Tenebrio molitor, and Dermestes vulpinus

Amino acid	Per cent of the total amino acid mixture		
	Rose *et al.*, 1948 19 amino acids	Rose *et al.*, 1948 10 amino acids	Almquist and Grau, 1944 20 amino acids
DL-alanine	2.54		3.33
L-arginine hydrochloride	3.18	4.20	4.67
DL-aspartic acid	2.54		6.67
L-cystine	1.27		1.33
L-glutamic acid	12.69		16.65
Glycine	.64		6.00
L-histidine hydrochloride	6.03	8.15	2.67
L-hydroxyproline	.63		.67
DL-isoleucine	10.16	13.95	6.67
L-leucine	7.62	10.40	6.67
L-lysine	9.53	13.01	4.67
DL-methionine	5.07	6.97	3.33
DL-norleucine			.67
DL-phenylalanine	7.62	11.40	3.33
L-proline	1.27		6.67
DL-serine	1.27		1.33
DL-threonine	8.89	12.10	10.00
L-tryptophane	2.54	3.49	1.33
L-tyrosine	3.81		6.67
DL-valine	12.70	17.35	6.67
$NaHCO_3$	8.07	11.00	5.00
Total	108.07	111.02	105.00

acids, and no carnitine. The results of these tests are given in Table II . *Dermestes* failed to grow on these diets, and *Tenebrio* grew very poorly. *Tribolium*, however, grew on Rose's 19 amino acid mixture as well as it did on casein. With only the 10 essential amino acids in the diet, growth was somewhat delayed. The Almquist mixture proved very much inferior. The experiments with *Tenebrio* and *Dermestes* were first started with first stage larvae. When these larvae failed to develop on the diets, the tests were repeated with larvae of larger size (*Tenebrio* larvae of about 20 mg. and *Dermestes* larvae of about 10 mg.). It was expected that larger larvae which had originally been grown on an optimal diet might be more robust and more able to survive and overcome any adverse effect of amino acid

TABLE II

Response of three insects to amino acid diets

Amino acid mixture and reference	*Dermestes vulpinus*	*Tenebrio molitor*	*Tribolium confusum*
19 amino acids Rose *et al.*, 1948	−	−	++++
10 amino acids Rose *et al.*, 1948	−	±	+++
20 amino acids Almquist *et al.*, 1944	−	±	++
Casein control diet	++++	++++	++++

++++ is growth equal to that on casein.
− is no growth.

diets. However, the larger larvae also failed to develop. All attempts to grow *Dermestes* and *Tenebrio* on amino acid mixtures have so far failed. The good results obtained with *Tribolium* on Rose's mixtures, however, were a starting point for further experiments.

B. The requirements of Tribolium for individual amino acids

Two of Rose's amino acid mixtures were used, one which contained 19 amino acids and another which contained only the 10 "essential" acids, in the proportion shown in Table I. A series of diets was then devised in which each of the amino acids was left out, one at a time. The results were clear cut. In every single case in which one of the 10 essential acids was omitted, the larvae failed to grow (Tables III and IV). Each of the remaining "non-essential" acids could be omitted from the diet, without noticeable effects (Table IV). However, larvae

TABLE III

*Effect on Tribolium larvae of omitting each amino acid from a diet containing the 10 "essential" amino acids**

Exp. 12—weighed at 15 days			Exp. 14—weighed at 20 days		
Diet	No.	Avg. wt. (mg.)	Diet	No.	Avg. wt. (mg.)
Casein control	18	0.55	Casein control	16	1.80
All 10 amino acids	18	0.40	All 10 amino acids	11	0.80
Without L-arginine	10	0.10	Without L-lysine	1	0.10
Without L-histidine	6	0.10	Without DL-methionine	0	
Without L-isoleucine	9	0.08	Without DL-phenylalanine	1	0.10
Without L-leucine	0	—	Without DL-threonine	0	
Without L-tryptophane	13	0.09	Without DL-valine	0	

* None of the larvae on deficient diets survived to pupate.

always grew faster in the presence of 19 amino acids than of ten, in spite of the fact that the total level of amino acids was the same in both instances (Table IV). Superior growth of rats on a mixture of 19 amino acids, as compared with the 10 essential acids, has previously been reported by Rose *et al.* (1948).

TABLE IV

Tribolium confusum. Effect of omitting each amino acid from a diet containing 19 amino acids (Rose diet XXIII)

Experiment 17

Omission or other variation	20 day larvae		Pupae		Adults	
	No.	Avg. wt. (mg.)	No.	Avg. time (days)	No.	No. normal
Casein	15	0.73	14	33.0	14	1
19 amino acids	20	1.11	20	30.1	16	3
No alanine	19	1.14	16	31.4	12	1
No aspartic acid	17	1.18	17	32.2	16	3
No cystine	19	1.17	18	30.9	18	2
No glutamic acid	19	1.11	15	31.4	11	1
No glycine	18	1.31	17	30.3	10	2
No hydroxyproline	12	1.09	10	30.7	9	0
No proline	18	1.00	16	32.7	13	0
No serine	18	0.84	15	32.0	14	3
No tyrosine	19	1.17	19	33.2	16	1
The 10 essential amino acids	18	0.53	12	41.5	11	1
			(Rose diet XXIV)			
			Experiment 16			
Casein	19	.96	18	30.7	15	0
19 amino acids						
Ground by hand, mortar	12	.93	9	34.2	8	0
Ground in ball mill—22 hrs.	17	.8	13	35.5	8	3
No arginine	5	.01	0	*	0	0
No histidine	4	.01	0		0	0
No isoleucine	4	.01	0		0	0
No leucine	3	.03	0	*	0	0
No lysine	3	.01	0	*	0	0
No methionine	9	.01	0	**	0	0
No phenylalanine	4	.01	0	**	0	0
No threonine	3	.01	0		0	0
No tryptophane	2	.01	0		0	0
No valine	7	.01	0	*	0	0

* One small larva at 90 days.
** Two small larvae at 90 days.

At the end of 8 weeks, when it was apparent that growth was not possible in the absence of any of the 10 essential amino acids and when most of the larvae had died, 1% yeast was added to each of the deficient diets and the experiment run again with a fresh lot of first stage *Tribolium* larvae. Growth was very much faster after the addition of yeast, and the effect of amino acid deficiencies was largely

obscured. This phenomenon is difficult to understand in view of the fact that the addition of 1% yeast only insignificantly adds to the total amount of certain of the essential amino acids in the diet.

Since a diet which contained 19 amino acids always proved superior to one with only 10 essential amino acids, an attempt was made to evaluate the effects of the non-essential amino acids in the diet. Omitting any single non-essential acid had no effect on the diet (Table IV).

It was then considered possible that the amino acids in the mixtures used in the tests might not have been present in optimal proportions. In fact there was no *a priori* reason for such an assumption to be true. Thus those differences in growth rate, which existed between a 10 and a 19 amino acid mixture, might possibly be due to changes in the total amount of some of the acids present. Accordingly, further

TABLE V

Response of Tribolium to D-amino acids in a medium of 19 amino acids

Substitution in diet	D-form substituted				L-form added*			
	20-day larvae		No. of pupae	Av. time to pupation (days)	20-day larvae		No. of pupae	Av. time to pupation (days)
	No.	Av. wt. (mg.)			No.	Av. wt. (mg.)		
Casein control diet	16	0.90	4	27.0				
Amino acid control diet	19	0.60	12	38.0				
D-arginine	6	0.04	0		11	0.66	10	35.0
D-histidine	9	0.05	0		13	0.40	2	38.0
D-isoleucine	0				16	0.30	2	43.0
D-leucine	0				18	0.3	3	41.0
D-lysine	18	0.67	7	34.5				
D-methionine	18	0.80	2	31.0				
D-phenylalanine	17	0.50	0					
D-threonine	0				14	0.30	6	46.3
D-tryptophane	1	0.05	0		15	0.40	4	38.5
D-valine	0				17	0.30	1	39.0

* The L-forms of the respective amino acids were added and the diets re-infested with larvae.

tests with 19 amino acids were devised in which the amount of each amino acid was doubled in individual tests. The hypothesis was that this procedure might produce two kinds of effects. If the diets had been improved, there would have been an indication that the original mixtures did not contain enough of certain acids for optimal growth. If the diets became worse, there would have been an indication that the basic mixtures already might have contained excessive quantities of certain acids. This experiment did not show any clear-cut changes in the efficiency of the diets. Poorer growth resulted with double amounts of aspartic acid and valine. Somewhat poorer growth also resulted when these amino acids were added to a casein diet. However, these effects were only slight.

In further series of tests the D-forms of the ten essential amino acids were individually substituted for the L- or DL-forms, in a diet consisting of 19 amino acids. The D-forms of arginine, histidine, isoleucine, leucine, threonine, trypto-

phane and valine were entirely inactive (Table V). It was considered possible that some of them might have been not merely inactive, but actually inhibitory. Consequently the diets with D-acids, on which the larvae had failed to grow, were later supplemented with the respective L-form and new larvae added. The larvae grew somewhat slowly on most of the diets, which might have been due to the age of the diets. The results, however, did not suggest that the D-forms were inhibitory.

In one experiment (Table V) the D-forms of lysine and methionine gave as good larval growth as the L-forms, but pupation was fairly good with D-lysine and very poor with D-methionine. D-phenylalanine also showed good growth, but no pupation occurred. In a repeat of this experiment (Table VI), in which carnitine had been added to the diets, D-lysine proved entirely inactive. D-methionine was as active, and D-phenylalanine almost as active as the respective L-forms. All through this test the larvae pupated well and the adults were normal in the presence

TABLE VI

Response of Tribolium to the D-forms of lysine, methionine and phenylalanine in a medium of 19 amino acids

Substitution in diet	20-day old larvae		Pupation		No. of adults	
	No.	Av. weight (mg.)	No.	Av. time (days)	Abnormal	Normal
Casein control diet:						
Carnitine absent	20	.83	20	29.4	17	3
Carnitine present	20	.76	20	29.6	—	20
Amino acid control diet:						
Carnitine absent	16	1.12	12	28.6	12	—
Carnitine present	19	1.68	19	26.1	—	19
D-lysine*	dead	—	—	—	—	—
D-methionine*	17	1.68	17	25.9	—	17
D-phenylalanine*	20	.89	16	29.2	—	16

* Carnitine present.

of carnitine. The result shows that the small number of pupae in the first test might have been, in part, attributable to the absence of carnitine. This, however, would not explain why D-lysine was fairly active in one, and entirely inactive in another test.

DISCUSSION

The results on the amino acid requirements of *Tribolium*, as reported in this paper, closely follow those previously reported for other insects. Lemonde and Bernard (1951), in their work with the same insect, *Tribolium confusum*, reached similar conclusions. They obtained some growth, and even pupation in the absence of either lysine, threonine, phenylalanine, methionine, isoleucine, arginine, leucine and tryptophane, although growth in all these cases was very slow. This may have been due to the presence, in the diets, of one half per cent of yeast. Moore (1946) demonstrated the necessity of the 10 essential amino acids in the nutrition of

a carpet beetle, *Attagenus* sp.; the effect of the non-essential acids was, however, not studied. The larva of the yellow fever mosquito, *Aedes aegypti* L. was shown to require glycine for normal growth in addition to the essential amino acids, plus tyrosine for normal pigmentation, and in addition, cystine for normal emergence (Golberg and DeMeillon, 1948). *Drosophila* seems to require, in addition to the 10 essential acids (Schultz *et al.*, 1946; Rudkin and Schultz, 1947) glycine and cystine (Hinton, Noyes and Ellis, 1951). Contrary to some of the aforementioned authors, we have never had any indication that *Tribolium* benefitted by the presence in the diet of cystine or glycine, nor did we find evidence of a toxic effect of Dl- or L-serine as had been reported for *Drosophila* (Hinton *et al.*, 1951).

Information about the nutritional value of the D-form of an essential amino acid has been so far lacking for insects. In the amino acid requirements of man for maintenance of nitrogen equilibrum, D-methionine was as effective as the L-form and D-phenylalanine showed partial activity. The D-forms of valine, leucine, isoleucine, threonine, lysine and tryptophane were inactive (Rose, 1949). In the nutrition of the rat it is generally agreed that the D-forms of tryptophane, phenylalanine and methionine show full or partial activity, while those of the remaining essential acids are inactive (Rose, 1938; Rose *et al.*, 1948; Nasset and Anderson, 1951). For *Tetrahymena* which, in addition to the 10 essential amino acids, also requires serine, the D-forms of methionine, lysine and arginine are active, that of leucine is inhibitory, and those of the remaining six acids are inactive (Elliott *et al.*, 1952). *Tribolium* utilizes fully or partly the D-forms of lysine, methionine and phenylalanine. It therefore appears that the D-methionine is utilized by *Tetrahymena*, *Tribolium*, the rat and man, D-phenylalanine by *Tribolium*, the rat and man, D-arginine alone by *Tetrahymena*, D-lysine possibly by *Tribolium*, and D-tryptophane alone by the rat.

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Summary

1. The larvae of the flour beetle *Tribolium confusum* have been successfully grown on diets which contain 19 amino acids or the 10 amino acids which are essential in the nutrition of the rat. The larvae of two other beetles, *Tenebrio molitor* and *Dermestes vulpinus*, failed to grow on similar diets.

2. *Tribolium* requires the following amino acids for growth: arginine, histidine, lysine, tryptophane, phenylalanine, methionine, threonine, leucine, isoleucine and valine.

3. On a mixture of 19 amino acids, which in addition to the above-named acids also contains glycine, alanine, proline, hydroxyproline, glutamic acid, aspartic acid, serine, cystine and tyrosine, growth is somewhat faster than in the presence of 10 amino acids.

4. Addition of any one of the non-essential acids to the mixture of the 10 essential ones has no marked effect. None of the amino acids exerted toxic effects when added to the diet in double amounts.

5. *Tribolium* utilizes fully or partly the D-form of methionine, phenylalanine and, possibly, lysine. The D-forms of the 7 remaining essential acids were entirely inactive, but did not show marked toxic effects.

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ANTIMITOTIC SUBSTANCES FROM OVARIES[1]

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In a paper published two years ago (Heilbrunn, Wilson and Harding, 1951), it was shown that a powerful antimitotic substance could be extracted from the ovaries of the common starfish (*Asterias forbesii*). This substance tends to exert a liquefying influence both on the cortex and on the interior protoplasm of marine eggs, and because it prevents the mitotic gelation in somewhat the same way that heparin does, we were led to believe that it might possibly be a heparin or heparin-like substance. However, we had very little direct evidence to support this belief.

Accordingly, we have been eager to find out what we could as to the nature of the antimitotic substance. The work is part of a broad general program in which we seek to establish that all living material contains substances which favor protoplasmic clotting and those which tend to prevent such clotting. It is now clear that the colloidal behavior of protoplasm is quite similar to the behavior of vertebrate blood; and if this is true, it would be logical to suppose that the anticlotting substances of living cells include heparins and heparin-like substances. For a detailed discussion of protoplasmic clotting and how it influences not only cell division but other vital processes as well, see Heilbrunn (1951, 1952a, 1952b).

The fact that we can obtain a potent antimitotic substance from the starfish ovary is perhaps not surprising, for the eggs in the ovary do not divide until they leave it. In our earlier paper (Heilbrunn, Wilson and Harding, 1951), it was suggested that "perhaps the ovaries of many organisms are rich in heparin-like substances." As will be seen later, this idea is apparently a fruitful one.

Materials and Methods

In general, extracts were prepared in much the same manner as in our previous work, except for the fact that instead of merely cutting up the ovaries, we homogenized them before extraction with acidified sea water. Specific details concerning the preparation of various individual extracts are given in relation to individual experiments. When the extracts were dialyzed, cellulose dialysis tubing was used. This was purchased from the Arthur H. Thomas Co. of Philadelphia, and they state that according to the manufacturer, the Visking Corporation of Detroit, the average pore diameter of the cellulose material is 24 Ångstroms. The purity of the cellulose is said to be very high, but it contains some glycerine and approximately 0.1 per cent sulfur. During the process of dialysis, the tubes were agitated on a shaking apparatus.

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In testing for antimitotic action, in most cases we used the eggs of a marine worm, *Chaetopterus pergamentaceus*. As in earlier works, the eggs were kept in a constant temperature bath maintained at a temperature of 21° C.

Protoplasmic viscosity tests were made with an Emerson-type centrifuge. For information about the use of the centrifuge in viscosity determinations, see Heilbrunn (1950), Wilson and Heilbrunn (1952).

Results

The substance we extract from starfish ovaries is presumably responsible for the inhibition of mitosis in the ovary. As is well known, as soon as the starfish eggs leave the ovary and enter sea water, the large nucleus of the immature egg, that is to say, the germinal vesicle, breaks down and the maturation divisions begin. In order to prevent the breakdown of the germinal vesicle and the subsequent maturation divisions, it is only necessary to leave the eggs in contact with the ovary. Thus in one experiment, the ovaries of a starfish were cut up in 25 ml. of sea water. Left in this sea water in the presence of the minced ovaries, only 2 per cent of the eggs showed germinal vesicle breakdown. However, with progressive dilution of the sea water which had been in contact with the ovary, there was a progressive increase in the percentage of germinal vesicle breakdown, so that when the original fluid was diluted 64 times, the percentage of germinal vesicle breakdown rose to 69 per cent. The inhibitor effect of the ovarian substance is largely reversible. As a matter of fact, this effect of ovarian substance both on maturation and cleavage divisions has long been known to students of marine eggs, and they commonly wash eggs two or three times before experimenting with them; that is to say, they pour off the sea water over the eggs and replace it with fresh sea water, and then repeat this operation several times.

Table I

Effect of starfish ovary extract on division of Chaetopterus eggs. Eggs exposed two minutes after fertilization

Dilution	Per cent cleavage
1/100	0
1/200	0
1/400	93
1/800	97
Control	97

In the work reported previously (Heilbrunn, Wilson and Harding, 1951), the extracts from starfish ovaries that we studied, when diluted to more than 1 part in 10, did not have much effect on Chaetopterus eggs. But in the extracts that were prepared from homogenized ovaries, a dilution of 1 to 200 was still effective. This is shown in Table I, which illustrates the effect on cleavage of one of our extracts. To prepare this extract, 100 ml. of acid sea water at pH 5.8 were added to 50 g. of starfish ovaries, and the ovaries were then homogenized in a Waring blendor. (The acid sea water was prepared as in our previous work.) The homogenate was centrifuged in a Sorvall centrifuge at about 15,000 g. and the resultant supernate was neutralized with 0.1 N NaOH so that its final pH was 7.0.

Nature of the antimitotic substance in starfish ovary extract. What is the nature of the antimitotic substance in the extract from starfish ovaries? If we knew that, then we might go ahead to discover various other antimitotic substances in the hope that one or another of them would be useful in the treatment of cancer. From the beginning, our suspicion has been that the potent substance in our extracts was a heparin-like compound. Let us summarize the old and new evidence in support of this opinion. Some of this evidence will later be presented in more detail by individual members of our group.

1. The extract from starfish ovaries is strongly metachromatic, just as heparin is. That is to say, the extract gives a reddish color with dilute solutions of toluidine blue. Tests for metachromasy are best made in calcium-free sea water or distilled water, for the calcium ions tend to prevent the metachromatic reaction.

2. The metachromatic reaction with toluidine blue disappears in the presence of protein, just as the metachromatic reaction of heparin disappears in the presence of protein (Kelly, 1951). If the crude extract from starfish ovaries is salted out with varying concentrations of ammonium sulfate, the activity appears in the globulin fraction and not in the albumin fraction. If now the globulin fraction is re-suspended in 0.3 molar sodium chloride solution and digested with trypsin, the resultant solution is metachromatic. This solution, after boiling to destroy trypsin, exerts a strong anticoagulant action on sheep plasma and it also prevents cell division in Chaetopterus eggs. Also if the re-suspended globulin fraction is dialyzed for 48 hours against 0.3 molar sodium chloride, a metachromatic reaction is obtained in the dialysate, and the dialysate is likewise effective in preventing cell division in Chaetopterus eggs. These facts will be discussed more fully by one of us (Dunn). The fact that the potent substance is able to pass through the dialysis sac indicates that it is not a substance of high molecular weight. Perhaps a correlation is to be found with the fact that, as Chaet (1952) has shown, ordinary heparin in solution can break down and yield substances capable of passing through a dialysis membrane and nevertheless capable of powerful physiological activity. Chaet's experiments, so far presented only as a preliminary note, will be published *in extenso* before long.

3. The active substance is heat-stable. Solutions containing it can be heated to 99.5° C. for 30 minutes and still retain their activity. On the other hand, when the substance is combined with globulin as a result of the salting out procedure described above, its activity is lost after exposure to a temperature of 80° C. for 20 minutes. (Following such inactivation, the active substance can no longer be separated off by dialysis.)

4. The activity of the starfish ovary extracts is destroyed by dilute solutions of periodate. Potassium periodate was added to potent extracts. Then the excess periodate was removed by dialysis. The control, containing extract without periodate, was dialyzed in similar fashion. The precipitates that formed were all removed by centrifugation and the potency of the extracts was tested on Chaetopterus eggs. The periodate was completely successful in destroying the antimitotic activity of the extracts. This is shown in Table II. These experiments are consistent with the idea that a polysaccharide is responsible for the activity of the extracts, but they do not constitute absolute proof of such an idea, for substances other than polysaccharides may also be destroyed by periodate.

TABLE II

Effect of potassium periodate on potency of starfish ovary extract

% periodate	% cleavage			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
0 (control-extract alone)	0	0	0	0
0.25	96	84		100
0.5	93	92	94	
0.75	98	95	96	
1	96	96		
Control (no extract)	99	96	98	100

5. The dialysis behavior of the extracts is similar to that of heparin. As already noted, Chaet (1952) has found that when preparations of commercial heparin are dialyzed, a potent substance keeps coming through the dialysis membrane. He believes that heparin is continually breaking down to produce more of this substance. Similarly, when starfish ovary extract is dialyzed, a substance passes through the membrane and this substance is strong in antimitotic properties. However, the material that remains in the dialysis sac also prevents cell division. Table III shows the results of an experiment on Chaetopterus eggs. Both the substance or substances remaining in the sac after prolonged dialysis and the substance that diffused through the sac into sea water completely inhibited cell division. The active substance is not completely removed by a single dialysis

TABLE III

The effect of dialysis on the potency of starfish ovary extract in preventing cell division. (The extract was first dialyzed against double its volume of sea water for 7 hours, then against running sea water for 9 hours)

	% cleavage	
	Exp. 1	Exp. 2
Contents of sac after dialysis	0	0
Dialysate	0	0
Control (dialyzed sea water)	99	99

TABLE IV

The effect of repeated dialysis on the potency of starfish ovary extract in preventing cell division. (The extract was dialyzed against an equal volume of sea water for 12 hours and the antimitotic effect of the dialysate tested. Then the contents of the sac were dialyzed against running sea water for 12 hours, following which the contents were dialyzed against an equal volume of sea water for 12 hours and the antimitotic effect of the second dialysate tested)

	% cleavage
Contents of sac (after 2 dialyses)	0
First dialysate	0
Second dialysate	0
Sea water dialysate	95
Second sea water dialysate	90
Control (sea water)	96

operation. This is shown in Table IV, which gives the data on an experiment in which a second dialysate was still strongly antimitotic and completely prevented the division of Chaetopterus eggs. In this experiment, control tests were made with dialyzed sea water, for it is sometimes found that dialysis tubes give off substances that have some slight antimitotic action. (This might well be expected from the fact that, as previously noted, the cellulose dialysis tubes consist of polysaccharide containing a little sulfur.) In another experiment, an active antimitotic substance continued to pass through the dialysis membrane after seven successive dialyses. It is quite possible that when a living cell is exposed to a heparin-like substance or to a combination of such a substance with protein, breakdown products of the heparin-like substance diffuse into the cell, whereas the components of larger molecular size remain outside.

6. When an active extract of starfish ovary is placed in a dialysis sac, carbohydrate diffuses through the sac into the surrounding fluid. This is shown clearly by chromatographic tests. The carbohydrate is a polysaccharide. Chromatographic tests of the dialysate also indicate the absence of nucleic acids and amino acids. Details of these tests will be published later (by Dunn).

7. The ultraviolet absorption spectrum of starfish ovary extract is similar to that of heparin. This is shown in Figure 1. In this figure, the open circles show the absorption spectrum of a 0.17 per cent solution of sodium heparinate, kindly supplied by the Upjohn Co. of Kalamazoo, Michigan. The closed (completely black) circles show the absorption spectrum of a 2 per cent solution of the globulin fraction of starfish ovary extract in 0.5 *M* NaCl. In the preparation of this fraction, 100 g. of starfish ovary were washed in 0.5 *M* NaCl for one hour to remove excess mucus; the washed ovaries were then suspended in 200 ml. 0.3 *M* NaCl and homogenized in a Waring blendor. Following centrifugation, the globulin fraction of the supernatant solution was salted out with half-saturated ammonium sulfate solution, and the precipitate was made salt-free by dialysis against distilled water. The triangles show the spectrum of a highly dilute solution of starfish ovary extract. This was prepared from an extract made by extracting 20 gm. of homogenized starfish ovary in 40 ml. of acid sea water (pH 5.8) and then neutralizing the resultant solution. This extract was then diluted with ordinary sea water until it was only 0.26 per cent of its original strength. The dilution was made in order to obtain a curve at about the same position on the graph at the heparin curve. The readings on the (Beckman) spectrophotometer were made by Dr. Lester Goldstein.

The general similarity of the three curves in Figure 1 is obvious, and although this similarity does not provide proof that the starfish ovary extract does actually contain a heparin-like substance, it is certainly consistent with such a view.

Indeed, no one of the seven arguments that we have presented is in itself very cogent, but taken as a whole they do indicate rather strongly that the starfish ovary extract contains a heparin-like substance and that this substance is responsible for its anticlotting and antimitotic activity.

Antimitotic substances in fish ovaries. In our thinking about the starfish ovary extract, we were bothered by two facts. In the first place, we knew of no evidence in the biochemical literature of heparin or heparin-like compounds splitting to form compounds of lower molecular weight capable of passing through dialysis membranes. Secondly, and this from a practical clinical standpoint is more important,

we were soon led to believe that the starfish ovary extract which had so drastic a liquefying action and so strong an antimitotic effect on invertebrate marine eggs, was rather powerless on vertebrate cells and tissues. The starfish ovary extract acts on starfish eggs, Chaetopterus eggs, eggs of the sea urchin Arbacia and eggs of the clam Spisula; but we found no very great antimitotic activity on frog eggs, and the antimitotic action of the extract on embryo mouse cells in tissue culture was much less than that of ordinary commercial heparin. [The studies on tissue culture cells were made by Carol Bocher and were presented by her as a Master's thesis (Bocher, 1952).] Moreover, although ordinary heparin, or a breakdown

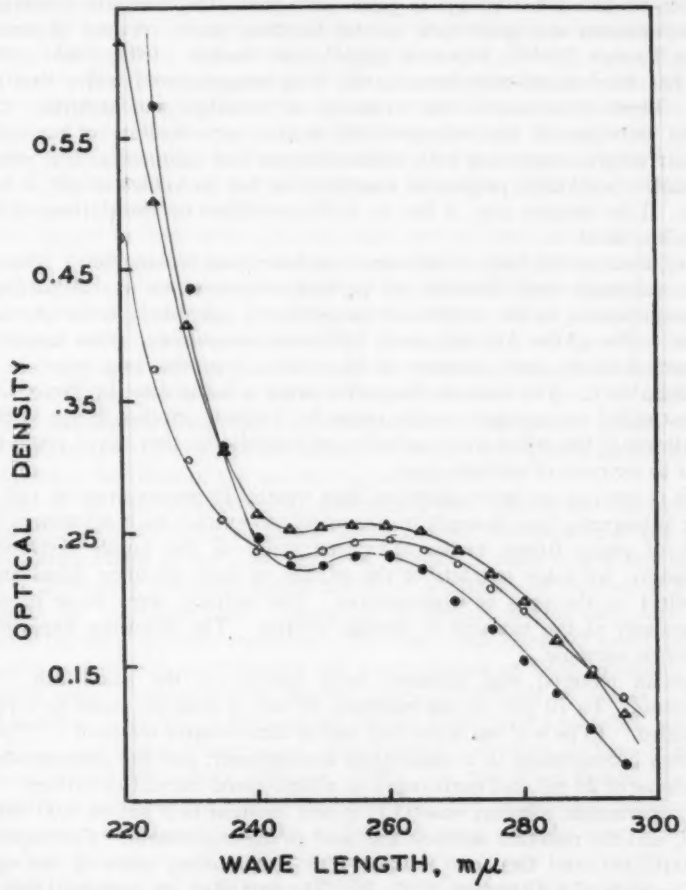

FIGURE 1. Comparison of the absorption spectra of heparin and two samples of starfish ovary extract. The open circles show the absorption spectrum of sodium heparinate. For further details, see text.

product of it, stops the frog heart in diastole (Kraus, Fuchs and Merländer, 1931; Chaet, 1952), starfish ovary extract has no effect on frog heart, although it does stop the clam heart in diastole. Also, in a few preliminary experiments, we found that starfish ovary extract had no obvious toxic action when injected subcutaneously into mice.

Fortunately, our attention was called to the fact that some fish of the family Tetraodontidae have in their ovaries a substance which has a very potent pharmacological and toxic action. This substance stops the heart of a toad or frog in diastole, and it thus acts like heparin, or rather like the breakdown product of heparin studied by Chaet (1952). Partly because of the toxic nature of this substance—it kills a few people in Japan each year—the literature concerning it is quite voluminous and goes back several hundred years. Useful papers include those of Tawara (1910), Ishiwara (1924), and Yudkin (1944, 1945). The substance has been called tetrodotoxin, and it is manufactured under that name in Japan. There it is used in the treatment of neuralgia and arthritis. Chemical study of tetrodotoxin has indicated that it is a carbohydrate of no very great molecular weight, containing both amino nitrogen and sulfur; also it is precipitated by alcohol. In all these properties, except for its low molecular weight, it resembles heparin. Like heparin also, it has an inhibitory effect on the clotting of bird and mammalian blood.

Here, then, is the kind of substance we have been looking for. Also, it is an ovarian substance and therefore of particular interest to us. Accordingly, we began experiments on the antimitotic properties of substances in the ovaries of the common puffer of the Atlantic coast, *Spheroides maculatus*. This fish belongs to the Tetraodontidae, and extracts of its ovaries stop the frog heart in diastole (Yudkin, 1945). The work on the puffer ovary is being done by Pierre Couillard, who first called our attention to the paper by Yudkin. Suffice to say at this point that extracts of the puffer ovary actually are antimitotic; they act in much the same manner as extracts of starfish ovary.

If it is true, as we have suggested, that ovaries in general may be rich in antimitotic substances, then it ought to be possible to extract such substances from the ovaries of many fishes, rather than just those of the family Tetraodontidae. Accordingly, we made extracts of the ovaries of some common fishes and tested their effect on the eggs of Chaetopterus. The extracts were made in much the same manner as the extracts of starfish ovaries. The following experiment will serve as an example.

Ovarian material was gathered from females of the small fish, *Fundulus heteroclitus*. To 10 gm. of this material, 10 ml. of acid sea water at a pH of 5.3 were added. (The acid sea water had had its bicarbonates removed.) The ovaries were then homogenized in a small glass homogenizer, and the homogenate diluted to a volume of 25 ml. and centrifuged in a high-speed Sorvall centrifuge. The pH of the supernatant solution was 6.15; it was brought to a pH of 8.00 with 0.1 N NaOH, and the resultant solution was used in the experiment. Chaetopterus eggs were fertilized, and then two minutes after fertilization, some of the eggs were placed in each of 4 dishes, *A*, *B*, *C*, *D*. The first dish, *A*, contained full strength extract; in *B* the extract was diluted with an equal volume of sea water, so that the resultant mixture was half strength. *C* had three parts of sea water for each

part of extract, and *D* had seven parts of sea water for each part of extract. Of the control eggs in sea water, 100 per cent gave off polar-bodies, but in *A*, polar-body formation was completely inhibited. In *B*, there was 12 per cent polar-body formation; in *C*, 27 per cent, and in *D*, 42 per cent. Thirty minutes after fertilization, at a time when it is known that the gelation has developed (Heilbrunn and Wilson, 1948), centrifuge tests were made rapidly with a hand centrifuge, as in previous studies from this laboratory. These tests showed that whereas the protoplasm of normal eggs had a viscosity well above the arbitrary value of 8 (and presumably, in accordance with earlier studies, about 14), the eggs in *A* exposed to the full strength Fundulus ovary extract had a protoplasmic viscosity of about 4. The eggs in *B* had a protoplasmic viscosity value of about 6. At 55½ minutes after fertilization, 50 per cent of the control eggs had cleaved, and a few minutes later, 97 per cent had cleaved. No one of the eggs in *A* ever cleaved, and only 25 per cent of those in *B*. The *C* eggs showed 27 per cent cleavage, and the *D* eggs 31 per cent. Some of the eggs from *A* and *B* were transferred to ordinary sea water. The eggs in *A* were badly injured, and following transfer to sea water they did not divide. When the eggs in *B* were transferred to sea water (after a 63-minute exposure to the extract), 53 per cent showed cleavage. Thus the effect of the extract is to some extent reversible.

Results similar to these were obtained with extracts from the ovaries of various other fishes. In all our studies with fish ovary extracts, we noticed that the active antimitotic substances tended to lose their potency in a relatively short time. Thus when the experiment with the Fundulus ovary extract was repeated a day later with the same extract, which had been kept overnight in a refrigerator, the effect both on the cleavage and on the protoplasmic viscosity was decidedly less. It should be noted that the extracts we use are very crude; no doubt in addition to anticlotting substances, they contain thromboplastic substances which promote clotting. When the extracts age, the effect of these thromboplastic substances may tend to override the effect of the anticlotting substances.

Because of the fact that powerful anticlotting and antimitotic substances are found not only in the ovaries of fishes of the family Tetraodontidae but also in ovaries of other fishes as well, we began to wonder if there might not be some evidence to indicate a pharmacological action or a toxicity of ovaries of fish not belonging to the Tetraodontidae. Literature in support of this idea does indeed exist, but it is very hard to assemble. For over four hundred years, scientific writers have commented on poisoning due to the eating of fish, but many of the articles that were written are in obscure journals, difficult of access. Gudger gathered together numerous references for Dean's bibliography of fishes, published in 1916–1923, and in the fifteen years following the collection of these references, he was able to find 180 others (Gudger, 1930). In the West Indies, there is a special word—*ciguatera*—that means fish poisoning; and in the East Indies and the South Seas, there is frequent reference to fish poisoning. Useful sources of information include papers by Taft (1945), Cohen, Emmert and Goss (1946), Vonfraenkel and Krick (1945), Gilman (1942), and Gudger (1918, 1930). Books by Phisalix (1922) and Pawlowsky (1927) may also be consulted. When men are poisoned by eating fish in the tropics, there is often uncertainty as to the

cause. Always there is a possibility that the fish may have spoiled; also there is an old superstition that fish become poisonous because they have eaten poisonous fruit. In the case of the barracuda, often found to be poisonous, it seems clear that only larger fish contain poison and then only at certain seasons of the year (Chisholm, 1808). This seems to indicate that the gonads may be involved. According to Coker (1930), the roe of garpikes (genus Lepisosteus) is said to be toxic, and in Germany it is well known that the ovaries of the barbel, a large cyprinid fish (*Barbus vulgaris*) are poisonous. According to McCrudden (1921), the ovaries of the pike are even more toxic than those of the barbel. Köhler, in 1933, writing in a magazine for practicing physicians, states: (p. 292) "Manche an sich ungiftige Fische geben zur Laichzeit unter nicht näher bekannten Verhaltnissen Ursache zur Vergiftungen." There is thus clear indication that fish ovaries may contain potent substances, substances which under certain conditions have a serious effect when ingested.

In fishes, the ovaries are not alone in containing substances that prevent the clotting of protoplasm. This is only to be expected if, as we believe, all types of living material contain anticlotting as well as clotting factors (see Heilbrunn, 1952b). We were not surprised, therefore, that when we extracted the testis of the toadfish (*Opsanus tau*) in the same manner that we extracted the ovary, we were able to obtain a substance that kept the protoplasm fluid and prevented cell division. As a matter of fact, in the Tetraodontidae, the testis is toxic as well as the ovary (Remy, 1883), and apparently contains the same type of substance that the ovary does. Also it will be remembered that the starfish testis contains much antimitotic substance (Heilbrunn, Wilson and Harding, 1951). In animals that breed only once a year, at times when little or no mitosis is occurring in the testis, this organ may presumably be rather rich in substances which prevent cell division. We found too that fish liver might also contain easily recognizable amounts of anticlotting and antimitotic substances—we used the liver of the angler or goosefish (*Lophius piscatorius*). This is to be correlated with the fact that the liver is a ready source of heparin and also with the fact that in Tetraodon the liver may be poisonous as well as the ovary (Tani, 1940). It is possible that the tetrodotoxin of the ovary is secreted in the liver.

The results reported in this paper provide additional evidence to show that many diverse types of living tissue, and indeed possibly all types of living cells, contain substances that prevent the clotting of protoplasm and exert a powerful antimitotic action. The ovaries of many animals are especially rich in such substances. Many of the anticlotting substances are either heparins or heparin-like substances. There is good reason to believe that the various substances vary widely both in molecular size and molecular composition.

At the present time the search for antimitotic substances in various organs and tissues of various organisms is being continued. There is undoubtedly a large and diverse group of naturally occurring heparin-like compounds which can act as anticlotting and antimitotic agents. Out of this large group of compounds, it should be possible to discover some which may be of real value in the treatment of tumors. More work is urgently needed. We need to know more about the chemistry of these heparin-like compounds, and their effect should be tested not only on simple isolated cells, but also on tumors.

Summary

1. Starfish ovaries contain a substance which prevents maturation divisions in the eggs contained in these ovaries.

2. By homogenizing starfish ovaries before extracting them with acid sea water, we have been able to prepare antimitotic extracts much more powerful in their action on Chaetopterus eggs than the extracts reported on previously.

3. There is additional many-sided evidence to indicate that the potent substance in these extracts is a heparin-like compound. Some of this evidence comes from chromatographic studies; also from studies of the absorption spectrum of the extract. Moreover, the potency of the extract disappears after treatment with periodate.

4. Ovaries of various species of fishes contain antimitotic substances which resemble in their action the substance or substances in starfish ovary extracts. In at least one family of fishes, the ovaries are known to contain a potent substance of heparin-like chemical composition and heparin-like properties.

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FURTHER INVESTIGATIONS ON THE INTERACTION BETWEEN SPERM AND JELLY-COAT IN THE FERTILIZATION OF THE SEA URCHIN EGG [1]

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The role played in fertilization by fertilizin, the sperm-agglutinating substance of egg water, was first emphasized by F. R. Lillie (1914). This substance has been shown (Tyler and Fox, 1939, 1940; Tyler, 1940) to be derived from the gelatinous coat of the egg and the evidence shows no other macromolecular constituents to be present in the latter. The gelatinous coat slowly dissolves as the eggs stand in sea water, providing thus the egg water of Lillie (1914), who had erroneously supposed its agglutinating property to be due to some substance actively secreted by the egg. Eggs deprived of their gelatinous coat are still fertilizable but require insemination with larger amounts of sperm (Tyler, 1941). In such eggs there is evidence (Tyler, 1941 *et seq.*) that a layer of fertilizin remains as a part of the vitelline membrane of the surface of the egg proper. It appears likely that reaction of the sperm with fertilizin on the surface of the egg is essential for successful fertilization to occur (see Tyler, 1948a, 1948b, 1949a, 1949b).

The well known fact that a jelly-coat solution causes sperm agglutination suggests already either a binding of jelly-coat molecules to the surface of the sperm or at least a modification of the sperm surface induced by the jelly-coat. The observation that the organic material of a purified solution of sea urchin jelly-coat can be practically completely adsorbed by sperm (Tyler, 1948b), speaks strongly in favor of the former interpretation to which the results presented in this paper give further support.

Our investigations started from the study of the mechanism by which the sperm are able to go through the jelly-coat in fertilization. An enzymatic mechanism was suggested by the finding of a jelly-coat splitting enzyme in extracts of sea urchin sperm (Monroy and Ruffo, 1947; Lundblad and Monroy, 1950; Vasseur, 1951). The activity of this enzyme was, however, weak. Furthermore its existence in some species of sea urchin was questioned (Krauss, 1950). Monroy and Tosi (1952), re-investigating the matter, interpreted their new results as being contrary to the early assumption of an enzymatic splitting and discussed the possibility of a quite different mechanism by which the sperm may be able to find its way to the egg through the jelly-coat.

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Summary of the experiments suggesting a non-enzymatic splitting of the jelly-coat by the sperm (Monroy and Tosi, 1952)

The previously mentioned conclusion on the non-enzymatic splitting of the jelly-coat by the sperm rested upon the following experimental evidence:

1. Living sperm added to a jelly-coat solution agglutinate and cause a sudden decrease of viscosity of the jelly. Irrespective of the quantity of sperm added, the viscosity drops in the course of about one minute and then remains constant. Washing of the agglutinated sperm with sea water does not restore their ability either to be re-agglutinated (see Lillie, 1914; Tyler, 1948b) or to "depolymerize" a fresh viscous solution of jelly-coat. That suggests that the reactive groups at the surface of the sperm have been blocked by the first reaction with the jelly-coat.

2. The interesting observation was recently made (Metz and Donovan, 1951) that fixed sperm can be agglutinated by jelly-coat solutions. We have also found that fixed sperm added to a solution of jelly-coat are able to "depolymerize" it just as well as living sperm do (for further details see Monroy and Tosi, 1952).

From these experiments it was suggested as a working hypothesis that in the reaction between sperm and jelly-coat, surface groups of the former react with groups in the jelly. As a result of this reaction fragments of the jelly-coat substance would become attached to the sperm surface. That would account for the apparent depolymerization of the jelly-coat.

The New Experiments

In order to develop further the analysis of the reaction between jelly-coat and sperm, two kinds of experiments were undertaken:

1. Attempts to recover the agglutinating factor from the agglutinated sperm
2. Quantitative estimation of some typical component of the jelly-coat substance before and after reaction with sperm

Material

Jelly-coat and sperm of *Arbacia lixula* were used throughout these experiments. Jelly-coat solution was prepared according to Tyler (1949b). Sperm were obtained by spontaneous shedding following cutting of the shells and were centrifuged once at 500 × g for 10 minutes in order to remove impurities and excess of seminal fluid. In our experience, washing with sea water prior to agglutination proved to be detrimental to the sperm.

1. Recovery of the agglutinating factor from agglutinated sperm

To a jelly-coat solution in sea water, sperm were added to saturation, *i.e.*, to the point when the solution had lost its agglutinating ability. The sperm were then centrifuged in the cold, washed twice with cold sea water and finally suspended in 4% formaldehyde at room temperature for one hour, being gently stirred from time to time. The suspension was centrifuged at high speed in the cold and the supernatant dialyzed against several changes of sea water and then tested for

agglutinating ability. Non-agglutinated sperm were treated similarly and the solution used as a control.

The extract from agglutinated sperm proved to have agglutinating power, whereas no effect whatsoever was obtained with the extract from non-agglutinated sperm.

Attempts to elute the agglutinating factor from agglutinated sperm by possibly a less drastic procedure (changes of pH, incubation at 37° C., salt solutions) were unsuccessful. In most cases, indeed, nucleic acid passed into the solution in rather large amounts and the solution was devoid of any agglutinating activity. A very small contamination with nucleic acid was actually found also in the formalin extract (small absorption peak in the U. V. at 260 mμ); we do not know whether this fact may be of any importance.

In the discussion following the presentation of these data by one of us (A. M.) at the Symposium on "The biochemical basis of morphogenesis" (Utrecht, August, 1952), Prof. Runnström objected that on account of the great ability of jelly-coat molecules to polymerize, especially under the influence of Ca-ions, it is possible that after a certain number of jelly-coat molecules have reacted with the sperm surface, other molecules may simply form a cloud around them. The recovered agglutinating ability may thus be due to the secondarily linked jelly molecules rather than to those which have become linked directly to the sperm surface.

The possibility of the formation of a cloud of jelly-coat molecules around the sperm, after the first ones have reacted with it, although difficult to imagine on the basis of our immunological knowledge, cannot be ruled out with certainty. This, however, does not invalidate the main fact that in the reaction between jelly-coat and sperm, jelly-coat molecules become attached to the sperm surface.

2. Importance of salts for the molecular architecture of the jelly-coat

The view that Ca-ions play an important rôle in the molecular architecture of the jelly-coat has been entertained especially by Vasseur (1949) who conceives the polysaccharide chains of the jelly-coat substance as being held together by Ca-bridges. Our experiments, however, in which the effect of addition of $CaCl_2$ on the viscosity of jelly-coat solutions was tested, have demonstrated that Ca causes a decrease of viscosity of the latter (Fig. 1). Much more dramatic is the effect of a 3.6% NaCl solution and of sea water, the former causing a drop of viscosity of 60% and the latter a drop of 70%. Urea, on the contrary, has no effect, thus suggesting that H-bonds play no part in holding together the molecules of the jelly-coat.

Furthermore there is another observation to which we would like to draw attention. The jelly-coat stains metachromatically with toluidine blue both *in vivo* and *in vitro:* as it is known this is a reaction given by all sulfonated polysaccharides. Now, *in vitro* this reaction is positive only if the jelly-coat is dissolved in distilled water whereas it is negative, *i.e.*, there is no metachromatic colour change, if the jelly-coat is dissolved in sea water, probably because under these conditions the $—SO_3$ groups of the jelly are present in a salt form. All this would suggest that the native jelly-coat must have a very low salt content, if any, and the $—SO_3$ groups are either free or linked to some anionic group by very weak bonds. Evidently

when the jelly-coat is brought into solution by the ordinary acid treatment the $-SO_3$ groups become free to react and they do in fact react with the ions of the sea water.

To the influence of salts is also probably due the swelling and dissolution of the jelly-coat of unfertilized eggs when standing in sea water.

3. Quantitative estimation of a component of the jelly-coat substance before and after reaction with sperm

In order to obtain further evidence for the attachment of the jelly-coat material to the surface of the sperm, experiments were performed to determine quantitative changes in some typical component of the jelly-coat as a result of its reaction with sperm. Analyses of sea urchin jelly-coat have proved it to be a hexosamine-free sulfonated glycoprotein (Vasseur, 1949, 1950; Tyler, 1948a, 1949a, 1949b). The

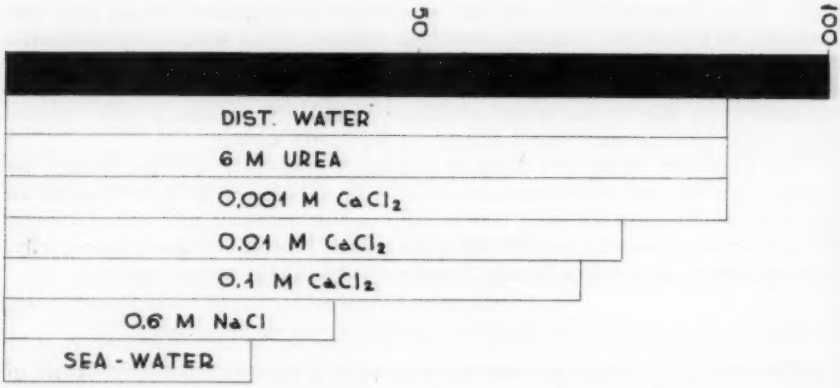

FIGURE 1. Effect of different salt solutions and urea on the viscosity of a jelly-coat solution in distilled water. The viscosity of the undiluted sample is taken as 100. To 3.0 cc. of this, 0.5 cc. of the solutions to be tested were added.

carbohydrate components are different in the different genera thus far studied (reviewed by Runnström, 1951). As to the jelly-coat of *Arbacia lixula*, fucose is by far its largest component, only small amounts of galactose being also present (Minganti, personal communication). Fucose estimation in the jelly-coat solution before and after reaction with sperm was therefore thought to be a good indication for the attachment of jelly-coat substance to the sperm. Such analyses are complicated by the fact that *Arbacia* sperm in sea water and in jelly-coat solution release a fucose-containing phosphate ester. The study of the latter phenomenon is being carried out by one of us (L. T.). The results of the analyses therefore had to be corrected for a blank in which sperm were suspended in sea water.

Experimental

In a typical experiment, to 1.0 cc. of jelly-coat solution in sea water, 2.0 cc. of sperm suspension were added and after 30 seconds the agglutinated sperm were

centrifuged off. The supernatant was collected quantitatively, brought to 4.0 cc. with sea water and 1.0 cc. of chilled 10% perchloric acid was added. The solution was kept in the cold for one hour and then centrifuged. The addition of perchloric acid was found to be necessary as otherwise sperm may remain in suspension and interfere with the colorimetric estimation of fucose. Sea water was substituted for jelly-coat in the blank for the fucose released by the sperm. The zero point, *i.e.*, the estimation of fucose content of the jelly-coat before reacting with the sperm, was done diluting 1.0 cc. of the jelly-coat solution to 4.0 cc. with sea water and then adding 1.0 cc. of perchloric acid. Aliquots of these solutions were used for fucose determination according to Dische and Shettles (1948). Three fucose standards were run with each analysis.

The results of these analyses are summarized in Table I.

Quite independently, similar results have been recently communicated in a short paper by Hultin *et al.* (1952).

These experiments conclusively indicate that the interaction between jelly-coat and sperm consists of a reaction in which surface groups of the sperm react with

TABLE I

Effect of the treatment with sperm on the fucose content of Arbacia jelly-coat solution

μg fucose/cc. in	
Jelly-coat control	Sperm treated jelly-coat*
234.0	44.0
9.8	4.2
120.0	49.0
19.8	2.2
42.0	25.6

* Corrected for the fucose released by the sperm in sea water.

and bind to some groups in the jelly. Now, Metz and Donovan (1951) have shown that alkylation of the $-NH_2$ groups in the sperm results in prevention of the agglutination by jelly-coat solution. That makes it highly probable that the reaction occurs between $-NH_2$ groups at the surface of the sperm and $-SO_3$ groups in the jelly-coat.

Model experiments

If the conclusion as to the groups entering in the reaction between jelly-coat and sperm is correct, it must be possible to duplicate the experiment using ion-exchangers having reactive groups similar to those of the sperm. Anionic exchange resins in fact have proved to be a satisfactory model. Most of our experiments were carried out with Amberlite IR 4B which is a weakly basic phenol-formaldehyde type resin with $\equiv N$ as an active group. Before use, the resin was blotted on filter paper to avoid dilution of the sample. Jelly-coat solution in distilled water was shaken with the resin and the viscosity was measured before and in the course of the experiment. As seen in Table II, treatment of the jelly-coat with the resin causes a drop of viscosity of the jelly. When the viscosity had reached that of water, a very low agglutinating titer was found and chromatography showed that the fucose spot had entirely disappeared, while the one of galactose was apparently

unchanged (Fig. 2). This different behavior of fucose and galactose is very peculiar and deserves further attention.

The resin used in the treatment of the jelly-coat solution was thoroughly washed with distilled water and eluted with 0.5–2% Na-bicarbonate or 5% ammonium sulphate. The eluate, after dialysis against distilled water and sea water, proved to be endowed with agglutinating ability.

That by this procedure the jelly-coat fraction adsorbed on the resin can be recovered almost quantitatively is demonstrated by the following experiment.

Two samples of 3.0 cc. of jelly-coat solution in distilled water were shaken with about 3.0 g. of resin. The supernatant was collected quantitatively, the resin washed three times with distilled water and the washings added to the first supernatant. Finally the material adsorbed on the resin was eluted as previously described and fucose estimated in the supernatant and in the eluate (Table III).

TABLE II

Effect of treatment with Amberlite IR-4B on viscosity, fucose content and agglutinating titer of Arbacia jelly-coat solution

% control	% treated	Fucose μg/cc. in		Agglutinating titer	
		Control	Treated	Control	Treated
1.52	1.0	present*	absent*	not tested	1/50
12.10	2.0	350.0	125.5	not tested	
2.48	1.01	present*	absent*	not tested	
4.23	1.04	136.0	1.0	>1/10.000	1/50
14.5	1.92	460.0	185.0	1/100.000	1/5000

* Qualitative estimate from paper chromatography.

CONCLUSIONS

The results of the present experiments suggest that in the reaction between sperm and jelly-coat substance, "molecules" of the latter adhere to the sperm surface. As previously mentioned, a similar point of view has been defended also by Tyler (1948b). Coating of bacteria by mucin is a well known phenomenon (reviewed by Olitzki, 1948). We think, however, that in this case not a simple coating but an actual chemical reaction occurs. Very likely the reaction is between the $—NH_2$ groups at the surface of the sperm and $—SO_3$ groups in the jelly-coat. This reaction may also account for the so-called depolymerization of the jelly-coat caused by the sperm. In a previous communication (Monroy and Tosi, 1952) it was suggested that as a consequence of this reaction the molecules of the jelly-coat would undergo fragmentation. As, however, nothing is known as to the length of the molecules of the jelly-coat before and after reaction with the sperm, it is difficult at present to decide whether a fragmentation actually occurs or the decrease of viscosity is due to the fact that *whole* jelly-coat molecules bind to the sperm surface.

Following the multivalence theory of antigens and antibody of Heidelberger (1939), Tyler thinks (1948b) that fertilizin molecules (*i.e.*, jelly-coat molecules) may each bind with two or more spermatozoa and each spermatozoon may in turn

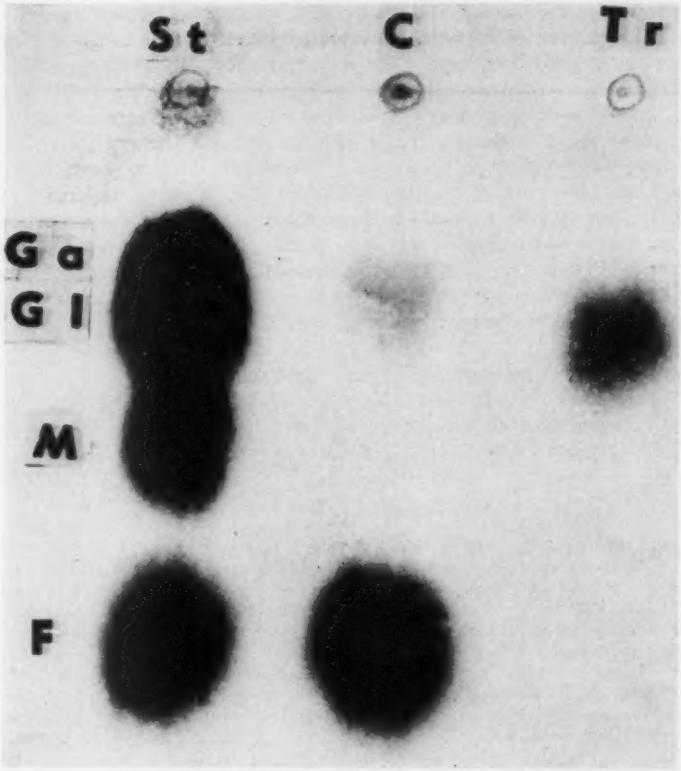

FIGURE 2. A chromatogram showing the disappearance of the fucose spot after treatment of a jelly-coat solution with Amberlite IR 4B(Tr). C = untreated jelly-coat. The greater intensity of the galactose spot in the treated sample is due to the higher concentration of the hydrolysate. St = standards of: Ga = Galactose; Gl = glucose; M = Maltose; F = Fucose.

bind to several fertilizin molecules. According to our findings, the jelly-coat in the living state should have a very low salt content, if any, whereas when dissolved in sea water its $-SO_3$ groups probably react and bind with the ions of the sea water. However, the jelly-coat in sea water not only agglutinates sperm but Vasseur has maintained (1949) that Ca reinforces the agglutinating power of the jelly-coat solution. That may be an indication that the type of bond that is estab-

TABLE III

Recovery of fucose after treatment of jelly-coat solution with Amberlite IR-4B

Control (untreated)	μg Fucose in Treated	
	Eluate	Supernatant
1002.0	119.0	850.0
91.0	20.4	65.6

lished between sperm surface and jelly-coat is different when the sperm cross the jelly-coat at fertilization and when they are agglutinated *by a solution* of jelly-coat.

When a spermatozoon crosses the jelly-coat surrounding the egg, it is coated by a halo of jelly-coat molecules which will not react with any other sperm and therefore a number of reactive groups of these molecules will remain free. Now the question arises whether the spermatozoon carries inside the egg its jelly-coat halo or the whole sperm surface is left outside. Should the former prove to be the case, then one could assume that the free groups of the jelly-coat molecules enter into reaction with the egg cytoplasm and this reaction may be of great importance either for the activation of the egg or for the reaction that establishes the block against polyspermy. In fact it is known that parthenogenetically activated eggs can be entered by a number of spermatozoa (reviewed by Monroy, 1953). Evidence on this point may be, however, hard to obtain.

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THE CHARACTER AND ULTIMATE FATE OF THE LARVAL SALIVARY SECRETION OF PHORMIA REGINA MEIG. (DIPTERA, CALLIPHORIDAE)

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The study of insect salivary glands has now attained a near classic interest, and diverse fundamental researches involving them have been stimulated in many fields. Fraenkel and Brookes (1953) have recently reviewed the literature concerning these glands in Diptera; especially the cytoplasmic changes occurring during the late larval period of *Phormia regina* and several species of *Drosophila*, together with the subsequent passage of cellular components into the lumen of the glands. Various functions have been assigned to this accumulated salivary material and a few postulations have been made regarding its disposition, but it is a noticeable fact that none of the previous workers has offered any experimental evidence as to the fate of the final larval gland contents. Fraenkel and Brookes (1953) have also described the manner in which *Phormia* and certain *Drosophila* orally release a fluid that flows along the underside of the insect, solidifies and securely attaches the newly formed puparium to the surface upon which it rests. By observing the comparative sizes of the salivary glands and investigating the volumes of glandular contents in the larvae before and after this secretion was released, they concluded from this indirect evidence that the secretion produced in the salivary glands was the same material which glued the puparium to its substrate.

The purpose of the present investigation was to collect the secretion from the late larval salivary glands and the material on the exterior of the puparium; to analyze them chromatographically in order to establish the identity of these products and to characterize it as well as the limited quantities would permit.

Methods

Laboratory cultures of adult *Phormia* were maintained on sugar and water. Eggs were collected on pork liver; the larvae were reared in battery jars with moist wood shavings and were furnished fresh liver daily. Prior to pupation, this species leaves its food supply, migrates through the shavings and evacuates its crop. This is referred to as the "empty-crop" stage in this work, and dissections revealed that during this period, the salivary gland lumen contains the greatest quantity of fluid attained throughout the larval life.

The salivary secretion was collected from empty-crop larvae by dissection in a modified Ringer solution (Ephrussi and Beadle, 1936). This was accomplished by cutting off the terminal third of the maggot with scissors, and then manipulating the insect with two pairs of fine forceps to turn it inside out over one of the points of the forceps. With the aid of a dissecting microscope, the glands which previously extended well into the abdomen were then readily discernible, floating free

in the saline, and could be easily teased free of the fat body. They were removed to a fresh solution of Ringer's to be freed of haemolymph, then quickly dipped into a dish of distilled water to remove the salts, and finally transferred to a clean, oversize slide (2 × 3″). Here the glands were punctured with a teasing needle and the secretion permitted to flow out onto the glass. The material from many insects was accumulated on a single slide and pooled by washing it off with distilled water. The volume was reduced in a vacuum desiccator over solid NaOH.

Full grown, empty-crop larvae were placed in petri dishes (about ten insects per dish) to pupate on a layer of clean sand. When the maggots pupated, the material which ordinarily flowed along the underside of their bodies and later anchored them now poured into the sand and merely aggregated a few sand grains at the anterior end of the puparia. By collecting the tanned puparia, carefully chipping off these small clumps of sand grains, pooling them and treating with warm water, the external secretion was extracted.

Hydrolysates were prepared by placing the secretions in small Pyrex tubes, evaporating to dryness in the desiccator, adding 2 ml. of 6 N HCl and sealing off the tubes. These were then steam-autoclaved for 18 hours at 15 lbs. pressure. After hydrolysis, the acid was removed by vacuum desiccation, distilled water added and completely evaporated four times to free the samples of HCl.

The hydrolyzed and raw secretions were analyzed by paper partition chromatography (Consden, Gordon and Martin, 1944), using the ascending modification of Williams and Kirby (1948). Two-dimensional chromatograms were prepared on Whatman No. 1 paper (9 × 11″), using phenol and water (80 gms. and 20 ml.) as the first phase solvent and a water-saturated mixture of equal parts of collidine and lutidine in the second phase. A 0.2% ninhydrin (in water-saturated butanol) spray was used to develop the colors. The final chromatograms were air dried and examined in transmitted light with the aid of a light-box viewer. Resultant spots were identified by, first, Rf values; second, cochromatography (an authentic sample of a known substance is added to the original application on the paper, intensifying the provisionally identified chromatographic spot which then acts as a single entity—behavior as such in different solvents presents strong evidence that the known and unknown are identical materials); and third, specific reactions for some of the components (to be described in a later section).

Results and Discussion

Four separate hydrolysates of the secretion collected from the salivary glands were prepared, and four from the material gathered from larvae which had pupated in the sand. The majority of these preparations were made from insects of different generations. In no cases was there any discrepancy in the number of spots on well prepared chromatograms, and the similar composition of these materials was repeatedly demonstrated by identical patterns consisting of the same components, present in constant relative intensities. The typical pattern, distribution and identification of spots are portrayed in Figure 1.

To test for free amino acids, unhydrolyzed preparations were chromatographed, and a single spot moved off the original site of application. This spot was present in both the glandular secretion and material collected from the puparia; but, when portions of these preparations were first dialyzed and then chromatographed, the

spot was absent. Cochromatography proved the free amino acid to be lysine. Chromatograms of dialyzed and nondialyzed materials, after hydrolysis, yielded lysine spots in each, but the nondialyzed preparations gave a more intense lysine spot. This indicated that, although lysine was present as a free amino acid, there was also lysine conjugated in the protein molecule.

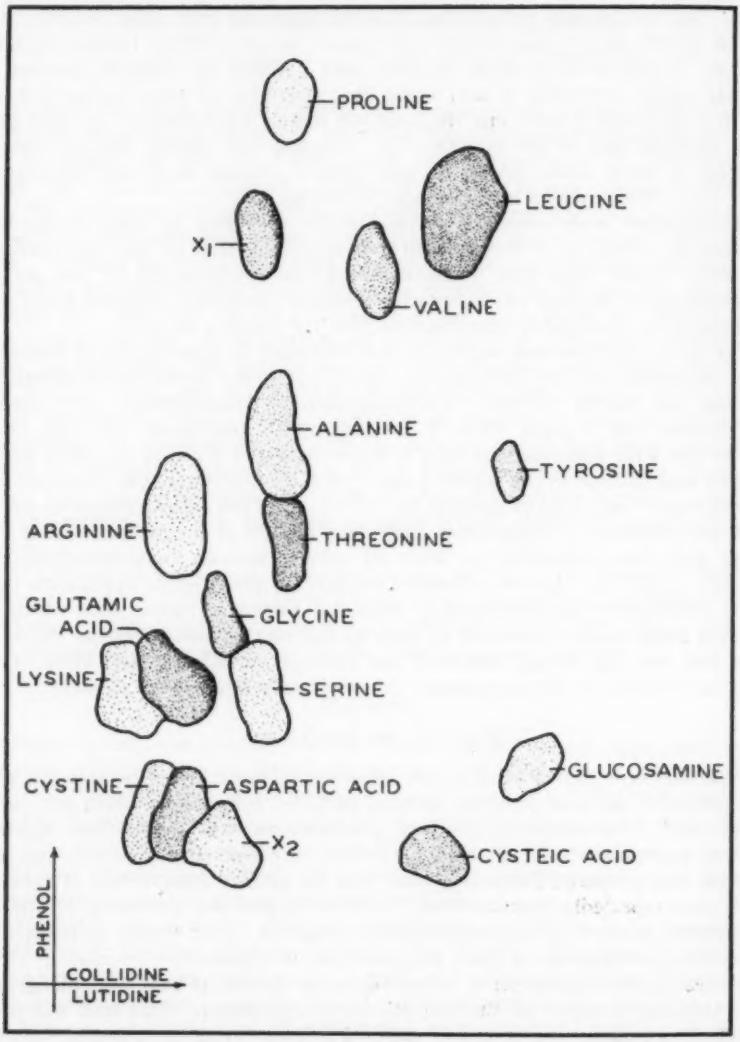

FIGURE 1. Chromatogram of hydrolyzed salivary secretion of larval *Phormia regina*.

Table I presents a complete list of the identified components found in this study together with a compilation of reported analyses made on dipterous salivary glands and associated products.

Cystine and its oxidation product, cysteic acid, appear brown when sprayed with ninhydrin, and both were present in the salivary material. Dent (1947) suggested that cysteic acid may be produced by secondary decomposition during chromatography; however, a sample of cystine hydrolyzed alone and treated as were the salivary products showed but a single cystine spot when chromatographed.

TABLE I

Comparison of chromatographic analyses of dipterous salivary glands and associated products

Component	Salivary gland chromosome *D. melano.* Yasuzumi and Miyao, 1950	Salivary gland chromosome *D. virilis* Blumel and Kirby, 1948	Salivary gland (*in toto*) *D. virilis* Blumel and Kirby, 1948	Salivary gland protein *D. melano.* Kodani, 1948	Salivary gland* secretion *Phormia*
Glycine	+	+	+	+	+
Alanine	+	+	+	+	+
Serine			+	+	+
Threonine			+	+	+
Valine	+	+	+	+	+
Leucine	+	+	+	+	+[1]
Tyrosine			+	+	+
Phenylalanine			+	+	−
Proline	+	+	+	+	+
Aspartic acid	+	+	+	+	+
Glutamic acid	+	+	+	+	+
Arginine	+		+	+	+
Lysine			+	+	+
Cystine					+
Cysteic acid				+	+
Methionine sulfoxide				+[2]	
Glucosamine				+	+
Unidentified			four		two
Free amino acids	none		none[3]		lysine

* Analysis of material from salivary gland and exterior of puparium.

[1] Leucine and/or isoleucine.

[2] Probably in error (Dent, 1948).

[3] LaCour and Drew (1947).

Cystine oxidized with 30% H_2O_2 was used for identification of the cysteic acid spots. Dent (1948) pointed out that by superimposing the peroxide directly on the hydrolyzed material on the paper, cystine would be quantitatively converted to cysteic acid. In chromatograms so treated, the cystine spot did not appear, and the cysteic acid spot was intensified. Further confirmation of cystine was accomplished by spraying the chromatograms with an iodine-azide spray reagent. As demonstrated by Chargaff, Levine and Greene (1948), the sulfur-containing amino acids were revealed as white spots against a brown background. After the location of the spot was marked, and the iodine faded, the paper could be resprayed with ninhydrin.

The spot labeled X_1 occupies the position ascribed by Dent (1947) to methionine sulfoxide and was later reported as such in the salivary secretion of *Drosophila* by Kodani (1948). When methionine was treated with 30% H_2O_2, mixed oxidation products of methionine sulfoxide and methionine sulfone resulted, and when this mixture was cochromatographed with the salivary secretion, X_1 was reinforced by methionine sulfoxide. However, neither adding the peroxide to the original material on the paper before chromatography, nor treating the final chromatogram with the iodine-azide reagent gave the anticipated reactions of a sulfur-containing amino acid in this position. Beta-aminoisobutyric acid has also been reported to have the same Rf values as methionine sulfoxide (Crumpler, Dent, Harris and Westall, 1951), but as this compound has never been identified as a protein hydrolysate product, it is unlikely that this amino acid is X_1.

A second unknown substance, labeled X_2, was characterized by a yellow ninhydrin reaction. Although this reaction is typical of the imino acids, X_2 does not migrate to the site occupied by any of the known substances of this nature.

TABLE II

Nitrogen composition of salivary products of Phormia regina

Product	Dry weight of material analyzed	Total nitrogen	Per cent nitrogen	Mean nitrogen per cent
Secretion collected from salivary glands	1.4 mg.	0.111 mg.	7.9	8.0
	3.6	0.289	8.0	
Secretion collected from puparia (in sand)	2.9	0.231	8.0	7.9
	3.4	0.261	7.7	
	4.8	0.382	8.0	
	4.9	0.388	7.9	
Secretion collected from puparia and dialyzed	7.3	0.732	10.0	10.0
	5.9	0.597	10.0	

A compound with a similar ninhydrin color and Rf values has been reported on chromatograms of free amino acids in potatoes (Dent, Stepka and Steward, 1947), and has also been found free in other dipterous tissues (unpublished data). In the salivary material, this unknown substance is undoubtedly conjugated in the protein molecule, as it appears only after hydrolysis.

In Table II, micro-Kjeldahl nitrogen determinations are compiled. These materials were collected in small aluminum-foil boats, and dried to constant weight before analysis. The nitrogen content of the fluid collected from the glands is in good agreement with that of the material emitted into the sand. The nitrogen values are higher in dialyzed samples, but are still unusually low for a typical protein. Kodani (1948), working with the glandular secretion of *D. melanogaster*, reported 10.8% nitrogen in samples exhaustively extracted with ether, and further demonstrated that a considerable quantity of crystalline, inorganic salt was present after hydrolysis and evaporation of HCl from the residue. He proposed that the low nitrogen figures could be accounted for by the presence of the salt together with a large amount of glutamic acid and glucosamine, both of which are low in

nitrogen. The *Phormia* products, when permitted to dry on glass slides, frequently crystallized in dendritic patterns which could be a result of the presence of inorganic salts. However, if this were the principle contributing factor to the low nitrogen figures, it would be expected that dialyzed samples would contain more nitrogen than the determined values of 10%. Lesher (1952) presented data suggesting that the substance synthesized by the larval salivary gland of *Drosophila robusta* is a conjugated protein composed of a protein bonded to a polysaccharide, *i.e.*, a mucopolysaccharide. This could possibly explain the low nitrogen figures obtained by Kodani and in this study.

Biuret and ninhydrin (triketohydrindene hydrate) tests were both strongly positive and indicative of the proteinaceous nature of the salivary gland secretion. The protein was water soluble and could be precipitated with hot or cold 10% trichloroacetic acid. Millon's reaction was positive, confirming the presence of tyrosine, and the xanthoproteic test also gave a strong reaction. The Hopkins-Cole test for tryptophane was slightly positive, but as only acid hydrolysates were prepared, the presence of this amino acid was undetected. Although glucosamine was demonstrated as a constituent of the protein, results of the Molisch test were doubtful. Specific carbohydrate tests, Benedict's, Barfoed's and Selivanoff's, were all negative.

Summary

1. In a comparative chromatographic study of the fluid in late larval salivary glands, and the substance which is responsible for adhering puparia of *Phormia regina* to their substrate, evidence is presented indicating that these materials are identical in nature and composition; this constitutes convincing proof that the "puparial cement" is the ultimate fate of the larval salivary secretion.

2. The identity of these secretions, collected from two different sites (the salivary glands and the exterior of the puparia), has been demonstrated by like nitrogen composition; the presence of a single free amino acid, lysine, in each fluid; the same components in the protein constituent (amino acids—glycine, alanine, serine, threonine, valine, leucine, tyrosine, proline, aspartic acid, glutamic acid, arginine, lysine and cystine; two unknown substances, and the carbohydrate, glucosamine); as well as by the reactions to several biochemical characterization tests.

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SIZE DISTRIBUTION, EROSIVE ACTIVITIES, AND GROSS METABOLIC EFFICIENCY OF THE MARINE INTERTIDAL SNAILS, LITTORINA PLANAXIS AND L. SCUTULATA [1, 2]

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The two common representatives in Southern California of the nearly worldwide genus, *Littorina*, are *L. planaxis* and *L. scutulata*. The former generally occurs at higher levels in the intertidal, but the zones of distribution of the two species overlap. *L. planaxis* may often be found 5 to 10 feet above spring high tide level, but *L. scutulata* prefers a zone two or three feet on either side of the high tide mark. Published information concerning these two species of snail is scanty in spite of their great abundance and their availability. Their importance to the high intertidal community, however, warrants an extensive study of their ecology, and the present paper describes some of the basic biology of these interesting animals.

Size Distribution

Inspection of colonies of *Littorina* at different places along the La Jolla shore has revealed that the majority of snails at any given locality fall within certain rather well defined size limits. Lysaght (1941) noted a similar condition in *L. neritoides* on the Plymouth Breakwater. In order to gain a more exact picture of size distributions in the present study, three typical *Littorina* environments were chosen, and height measurements were made of all the periwinkles found within a selected area, representative of the environment.

Environmental description

The three environments are shown in Figure 1. The first (Fig. 1a) is a group of pools at Whale View Point in La Jolla. The area is subjected to vigorous wave action as even moderate tides. The second environment (Fig. 1b) is the seaward edge of the top of a broad shelf of rock that extends out from a cliff about 1/4 mile north of the Scripps Institution of Oceanography. The top of Shelf Rock is 1 to 1½ meters above spring tides, but the area under discussion is well splashed at high tide and a large wave may occasionally wash over it. The

[1] This work was supported by a research grant from the Rockefeller Foundation and represents a portion of a thesis submitted in partial fulfillment of the requirements of the Ph.D. degree, University of California. The author would like to express his gratitude to Professor Denis L. Fox for his continued interest and friendly supervision, and to Dr. Arthur L. Kelly and Messrs. James S. Kittredge and John R. Maher for helpful assistance.

[2] Contribution from the Scripps Institution of Oceanography, New Series No. 685.

third environment (Fig. 1c) is a protected portion of Shelf Rock some three meters removed from the second environment and splashed only occasionally by large waves.

All three localities are sedimentary sandstone, but the grain size is much finer at Shelf Rock. By exerting pressure the point of a knife may be forced into

FIGURE 1. Photographs of the three environments studied. *a*. Whale View Point; arrow points to typical tidepool from which snails were gathered. *b*. Pools of Shelf Rock; arrow points to region studied. *c*. Dry area of Shelf Rock; Region studied enclosed by white line and marked with arrow labeled 3: arrow labeled 2 points to second environment, about 3 meters away.

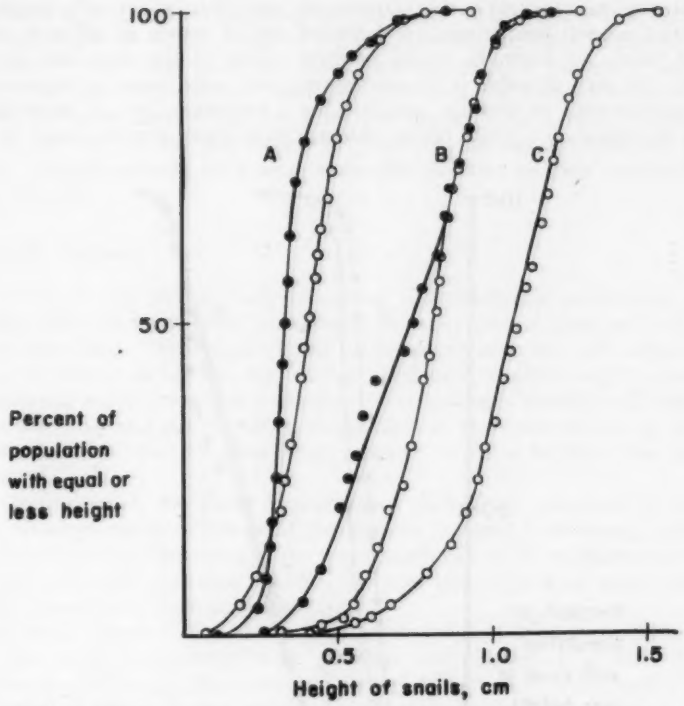

FIGURE 2. Cumulative curves for the *Littorina planaxis* populations of three environments. A. Whale View Point; B. Shelf Rock pools; C. Dry area, Shelf Rock. Measurements made in August, 1951, shown as circles; measurements made in July, 1953 shown as dots.

the sandstones to a depth of about half an inch. The top layers of sand particles may easily be removed by scraping. The topography is characteristically very irregular, great numbers of pools, depressions, basins, and small holes being present.

Results of measurements

The size distribution of *L. planaxis* and *L. scutulata* in each of these environments is shown by means of cumulative curves in Figures 2 and 3. The greatest dimension, "height," was measured, being the distance from the tip of the spire to the lower lip of the aperture. It can be seen that the populations at Whale View Point are rather small, averaging about 0.4 cm. The snails in the pools of Shelf Rock are generally intermediate in size, approximately 0.8 cm., while those on the sheltered dry area of Shelf Rock, although more heterogeneous than the other groups, are generally the largest, averaging almost 1.1 cm. in height. In the latter environment there were insufficient numbers of *L. scutulata* to enable the construction of a reliable curve.

In attempting to explain the variation in size distribution with locality, two possibilities suggest themselves. A favorable set of spawn in different areas at different times, for example, would produce curves of the type that have been obtained. Equally plausible is the contingency that some factor or factors operate in the environment to produce selection for a particular size of periwinkle. If the first hypothesis is correct, curves obtained from measurements made at a later

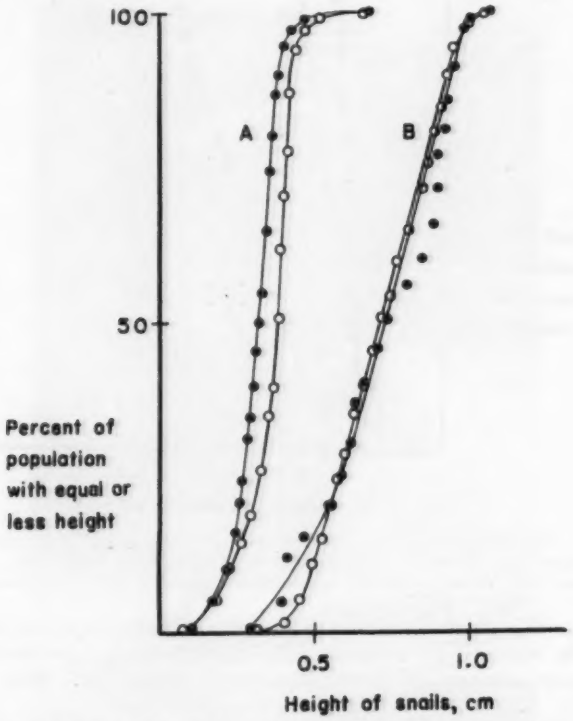

FIGURE 3. Cumulative curves for the *Littorina scutulata* populations of two environments. A. Whale View Point; B. Shelf Rock pools. Measurements made in August, 1951, shown as circles; measurements made in July, 1953, shown as dots.

date should be shifted towards the right along the abscissa; that is, effects of growth should be evident. If the latter hypothesis is the proper explanation for the phenomenon, the position on the abscissa of a curve should remain constant with time. Figures 2 and 3 show cumulative curves for populations in the first and second environments, constructed from measurements made about two years after the data discussed above were obtained. Measurements were not attempted for the third environment as appreciable numbers of large periwinkles had been permanently removed from this locality for other experiments. The expected

height increase of *L. planaxis* during this time can be calculated from data given below, and would be of the order of 0.5 cm., but the two sets of curves occupy approximately the same position. Environmental factors, therefore, appear to determine the size distribution of snail populations at any particular locale.

It should be mentioned that the three environments were under constant observation over the period that intervened between the measurements of Figures 2 and 3. The populations were never noticeably different in their size distribution during this time.

Factors affecting size

Wave action and salinity may be among environmental mechanisms capable of causing selection for a given snail size. In many places these two factors are inversely correlated. The higher levels of the spray zone are not wetted by the ocean so frequently as are the lower levels, and have therefore more opportunity for undergoing evaporation and achieving correspondingly greater salinities. The water of the pools and the surface moisture films of the lower levels, on the other hand, are renewed often by waves, and salinities at these localities are therefore not elevated.

As already noted, the three environments studied are subjected to different degrees of wave action. The snail populations composed of larger individuals appear to occupy the drier areas of the spray zone. In order to determine whether water currents might be more effective in removing large than small snails the following experiments were performed.

Four small (approx. 0.6 cm.), four intermediate (0.9–1.2 cm.), and four large (1.4–1.6 cm.) specimens of *L. planaxis* were placed on a small flat rock in a tidepool. When all the snails had emerged from their shells and were observed creeping, the rock was moved rapidly through the pool, creating a water current across the shells. After several vigorous swings through the water, only three small snails were left. A repetition of the experiment using six small, four intermediate, and four large snails ended with only four small snails remaining attached to the rock. When twelve medium-size *L. scutulata* (approx. 0.9 cm.) were placed on the stone with three small and two large *L. planaxis* for comparison purposes, the large specimens of the latter species were washed off with relative ease. After much effort three of the *L. scutulata* were eventually swept away, leaving nine of this species and the three small *L. planaxis* when the experiment was discontinued.

When a stream of sea water from a hose was allowed to play against the shells of *L. planaxis* creeping on a flat rock, large individuals were washed off more readily than small ones, and a snail of a given size was removed with greater ease when the stream was directed against the posterior part of the shell.

In order to gain an idea of the current velocities necessary to dislodge a snail, specimens of *L. planaxis* were placed in a Plexiglass tube of 3.5 cm., inside diameter, and after the animals had emerged from their shells and were crawling on the surface of the Plexiglass, the tube was gently filled with sea water. A current of known velocity was then allowed to flow through the tube and notations were made of the ability of the snails to remain attached for 10 seconds. The results

are given in Table I. A total of 10 large and 8 small snails was used in the experiment, and 6 trials were conducted.

Several hypotheses offer plausible explanations for the ability of small *L. planaxis* to withstand currents that remove large individuals. Physical factors might include greater water friction acting as drag on larger shells, or frictional forces slowing the current in the vicinity of the rock-water interface [3] thus favoring smaller snails. Biological factors might include loss of vigor and of tenacity with increasing age, or a disproportionate growth of the various parts such that the sole of the foot does not increase as rapidly as the surface area of the shell. Figure 4 shows that the last hypothesis is not supportable, hence one or more of the other factors may contribute to the distribution phenomenon.

TABLE I

Effect of current velocity on two size groups of Littorina planaxis

Size group	Velocity of current flowing over snails meters/second	Fraction of snails removed by current per cent
Large snails (1.3 to 1.6 cm.)	3.4	90
	2.3	90
	2.0	80
Small snails (approx. 0.7 cm.)	3.4	50
	2.0	50

The absence of large snails from areas exposed to vigorous wave action may thus be explained, but account has not yet been taken of the absence of small snails in the high, dry areas of the spray zone. The solution to this problem may lie in physiological age changes rendering the animal more capable of coping with exposure to air or to more variable salinity conditions. Salinity has been shown to influence shell size and shape in *Littorina* (Thorson, 1946; Agersborg, 1927) and further study along these lines will be necessary before a complete explanation of the observed size distribution can be proposed.

EROSIVE ACTIVITIES

Many intertidal animals bring about erosion of rock. *Littorina* species often occur in small basins above high water mark (Fischer-Piette, 1932; Clench, 1938; Lysaght, 1941) and have been credited by some investigators (Brunelli, 1928; Welch, 1929) with the production of depressions in rock. The snails feed by applying a file-like ribbon, the radula, to the substratum and transfer bits of it to the mouth by a scraping action. On the cliffs around La Jolla the animals scrape algae and fine detritus from the rocks and at the same time remove particles of the rock itself. Because of their great abundance, erosion resulting from their feeding is believed to be appreciable, and a quantitative estimation of the magnitude of the erosion will be useful in determining its importance with respect to other erosive processes.

Since the animals may not behave as they do in nature when kept in the laboratory over long periods of time, it seemed wisest to obtain as much data

[3] An idea for which I am indebted to Professor Roger R. Revelle.

as possible from snails in their normal environment. The simplest means for accomplishing this appeared to be to determine the number of times daily the gut contents are completely renewed, and also the proportion of the gut contents that is inorganic matter. The product of the two quantities would yield the daily rate of erosion.

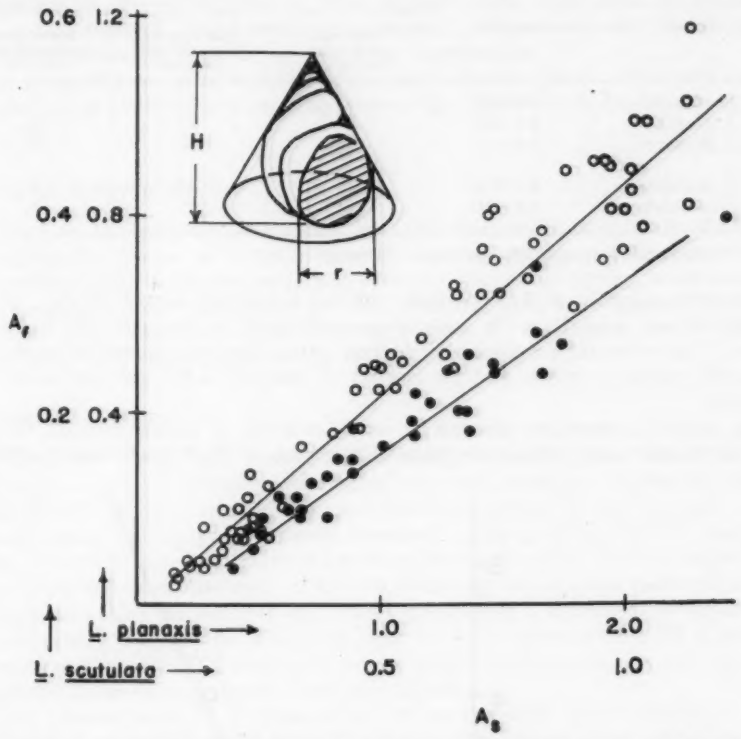

FIGURE 4. Relationship of shell surface to surface of the sole of the foot for *Littorina planaxis* and *L. scutulata*, indicating that the increase in area of each with age is proportionately the same. The sole area (A_f) was measured while the animals were crawling up the sides of a glass vessel. Shell surface (A_s) was arbitrarily taken as $1/\pi$ of $\pi \sqrt{r^2 + H^2}$, the formula for the curved surface of a right cone.

Time required to renew material in the gut

The number of times daily the gut contents are renewed will depend on the rate at which material is passed along the gut, which in turn may vary with the amount of time the snail is able to feed. Dissection of specimens of *L. planaxis* taken both from pools (46 specimens) and from areas that become dry at low tides (30 specimens) revealed that throughout the day the intestines of the former group were always full of unconsolidated material, whereas the intestines of the latter

TABLE II

Summary of an experiment to determine the time required for Littorina to cycle food completely through the gut; May 18, 1953; water temperature range 17° to 30° C.; 20 snails per size group

Species	Height cm.	Time elapsed until		
		First blue fecal pellet observed, hr.	Half of group defecated blue feces, hr.	Entire group defecated blue feces, hr.
L. planaxis	0.4–0.5	1¾	2	3
L. planaxis	0.7–0.95	2	3¾	5
L. planaxis	1.3–1.6	2¼	4¾	–*
L. scutulata	0.3–0.45	1¾	2	3
L. scutulata	0.8–0.95	2¼	4	5

* Thirteen had defecated blue feces after 7½ hours.

group were generally at least partially full but sometimes almost empty. It was concluded that animals in the pools graze sufficiently to keep the gut full at all times. Snails feeding on areas that become dry at low tide appear to graze only when the surface is moist, and the amount of material in the gut consequently is variable.

In order to determine the rate at which material is passed through the gut, groups of snails were allowed to graze on a section of Shelf Rock stained with the

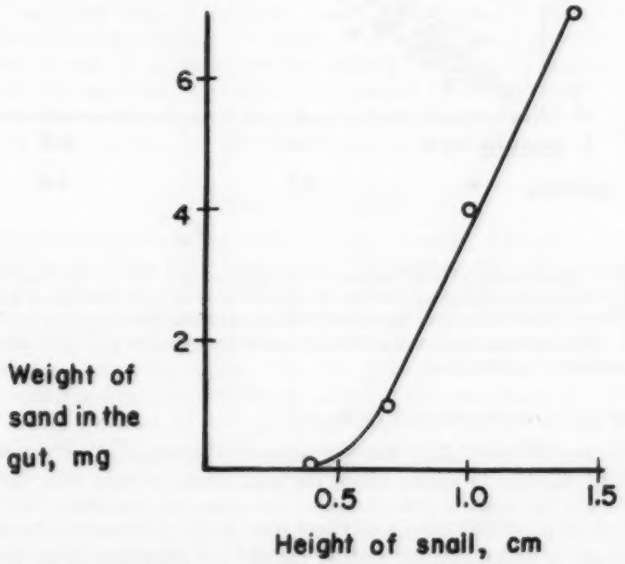

FIGURE 5. Relationship of sand contained in the gut to height of *Littorina planaxis*.

harmless dye methylene blue. After a period of an hour and a half had elapsed, the animals were removed to an unstained area and carefully observed for the first appearance of blue fecal pellets. While they were on the stained and unstained rock, the periwinkles were washed with fresh sea water every 10 minutes, and their behavior during the experiment seemed normal. Five such experiments were conducted on two spring and on three summer days. For snails of height 0.8 cm., cycling times of 2½ to 6 hours were obtained. The results were always similar and are described in Table II for one of the experiments.

A typical 0.8-cm. snail in a Shelf Rock pool, therefore, feeds sufficiently to keep the gut full at all times and probably renews the material completely four to eight times daily.

Inorganic matter in the gut

Sand in the gut was determined by extracting snails from their shells, incinerating the bodies in a tared crucible, cooling, adding a little concentrated hydrochloric acid to dissolve body ash, carefully decanting, rinsing, re-incinerating and weighing. Four size groups of 50 *L. planaxis* each were thus analyzed and the results are presented in the curve of Figure 5. An 0.8-cm. snail therefore contains on the average about 1.6 mg. of sand in the gut.

Calculated rate of erosion

Taking the Shelf Rock region, and considering the simplest case of a snail feeding in a pool, an estimation of erosion may now be made. It has been shown (Fig. 2) that the population in this environment averages 0.8 cm. in height. The average amount of inorganic material contained in the gut of an 0.8-cm. snail was found to be 1.6 mg., and, if we take the most conservative cycling period of 4 times daily, we obtain the value of $1.6 \times 4 = 6.4$ mg. of inorganic material passing through the gut of the snail per day. This presumably is equivalent to the amount of rock eroded daily. The density of Shelf Rock is approximately 2.5 g./cc. and calculations show that 100 snails, 0.8 cm. in height, would be capable of excavating a basin of 86 cc. yearly, almost a liter in a decade.

The concentration of *L. planaxis* in the environment under discussion is of the order of one snail per 30 cm.^{2} Erosion by this species alone, therefore, may be calculated to deepen the pools one cm. every 40 years. If *L. scutulata* is considered, the snail concentration increases to one snail per 12 cm.,^{2} and assuming that 0.8-cm. individuals of both species have similar feeding rates and weight of gut contents, erosion by both *Littorina* species combined will deepen the pools one cm. every 16 years. The snail concentration has remained fairly constant over the two-year period that is covered by these observations.

Comparison with erosion from other sources

Other erosive processes acting on the sandstone rocks of this area have been studied by Emery (1941, 1946). For exposed surfaces a general erosion rate of one cm. every 20 years was estimated, while for pools at Whale View Point nocturnal pH changes at low tides were calculated to cause a deepening of the

pools by one cm. every 33 years. The different processes causing rock erosion are probably additive in some cases and in other instances facilitate each other and are not additive.

Gross Efficiency

The average, gross metabolic efficiency, or the ratio of ingested food to organic matter incorporated as living tissue, may now be estimated for *L. planaxis* with the aid of additional data that have been obtained.

Growth

Growth rates were determined by measuring the increases in height of marked specimens of *L. planaxis* on Shelf Rock at various intervals for a period of a

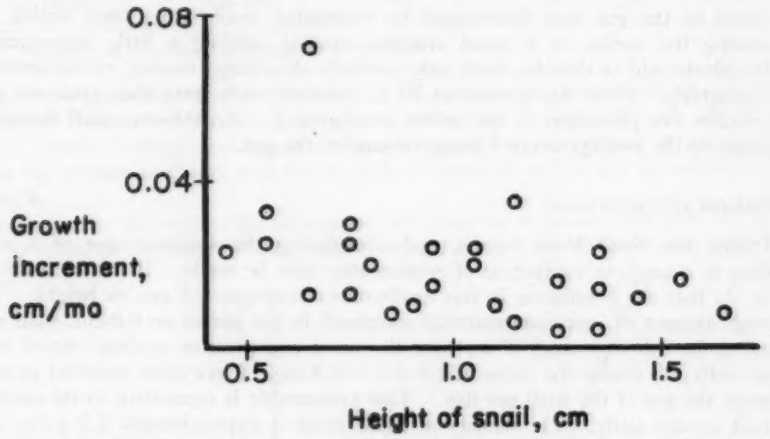

Figure 6. Twenty-six monthly growth increments observed in specimens of *Littorina planaxis* at Shelf Rock.

year and a quarter. The methods of converting an increase in spiral length to a height increase, used by Moore (1937) and Lenderking (1951) with other littorines, seemed to be complicated in the present case, since the apex angle of *L. planaxis* varies considerably, and it therefore appeared simplest to measure height increments over a long period of time. Figure 6 illustrates the results of the experiment, showing the growths of positively identified individuals from the snails that were recovered, out of a total of 300 originally marked. It may be noted that an 0.8-cm. snail has an average height increment of about 0.02 cm. per month.

To convert this value to an increase in tissue weight the relationship between snail height and dry tissue weight was obtained and is shown in Figure 7. Specimens of *L. planaxis* were dried in a vacuum oven for 24 hours at 80° C. and 460 mm. pressure. After cooling in a desiccator, the shell and dry tissue were weighed and the shell weight was obtained after removing the dry tissue by a half hour's immersion in boiling 20% KOH. Dry tissue weight was then readily calculated

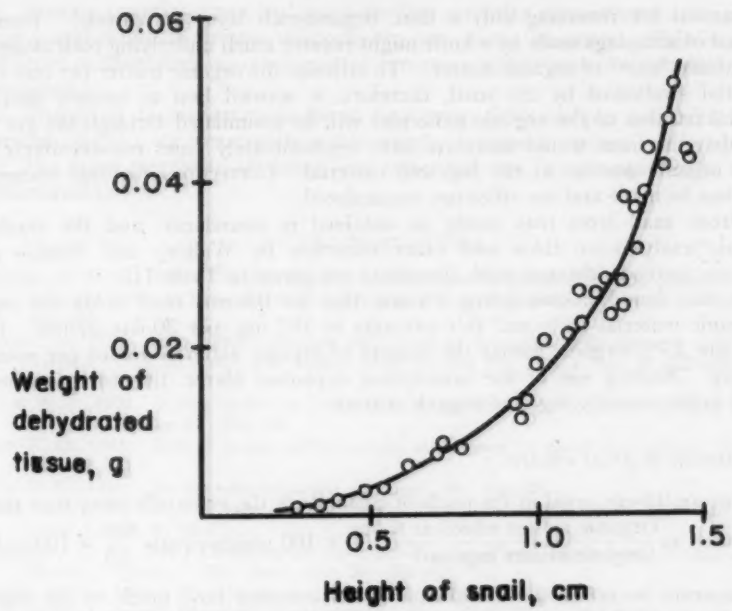

FIGURE 7. Relationship between dry tissue weight of *Littorina planaxis* and height.

by subtraction. Computations using Figure 7 indicate that an 0.8-cm. snail growing 0.02 cm. per month would have an increase in dry weight of 0.6 mg. for a like period.

Ingestion of organic matter

Determination of the amount of food ingested monthly could be readily accomplished if the organic matter per cent of the material swallowed by the snail were known. In scraping the surface of rocks, however, the snail has an excellent

TABLE III

Organic content of various materials

Material analyzed	Organic matter per cent	Average
Snail feces	2.4	
	2.0	
	3.0	
	3.2	2.7
Light manual knife scrapings of rock surface	2.4	
	2.6	2.5
Loose sand from tidepool bottom	0.8	
	1.0	0.9

mechanism for removing only a thin, organic-rich layer of material. Even the lightest of scrapings made by a knife might remove much underlying rock along with the surface "skin" of organic matter. To estimate the organic matter per cent of the material swallowed by the snail, therefore, it seemed best to assume that only a small fraction of the organic molecules will be assimilated through the gut wall, and that the feces would therefore have approximately (and conservatively) the same organic content as the ingested material. Corrections for this assumption can then be made and the efficiency recalculated.

Fresh snail feces may easily be obtained in abundance, and the results of organic analyses on these and other materials by Walkley and Black's rapid titration method (chromic acid digestion) are given in Table III.

It was found in calculating erosion that an 0.8-cm. snail voids 6.4 mg. of inorganic material daily and this amounts to 192 mg. per 30-day month. If the feces are 2.7% organic matter the amount of organic material voided per month is 5.3 mg. Making use of the assumption explained above, this is approximately equal to the monthly ingested organic matter.

Calculation of gross efficiency

For an 0.8-cm. snail in the pools of Shelf Rock the gross efficiency may now be estimated as $\frac{\text{Organic matter added as tissue}}{\text{Organic matter ingested}} \times 100$ which equals $\frac{0.6}{5.3} \times 100 = 11\%$.

In order to refine the calculation let us consider how much of the ingested organic matter might reasonably be assimilated across the gut wall from the food, of which some is built into new tissue. There must also have been assimilated a sufficient quantity of material to account for organic matter lost in respiration, and since the animals are poikilothermic and slow in movement, it would seem that this loss should be less than 4 times the amount added as new tissue. Taking the latter, then, as 20% of the total assimilated, and all other losses combined as 80%, and knowing that the animal adds 0.6 mg. per month as new tissue, we have 3 mg. per month as an estimate of the amount of organic matter assimilated across the gut wall. Recalculating the efficiency gives a value of $\frac{0.6}{5.3 + 3.0} \times 100 = 7\%$.

It will be recalled that in calculating erosion the time for cycling food through the gut was taken conservatively as 6 hours. The average value might be less, which would increase the erosion rate and depress the efficiency.

Summary

1. Size distribution curves for populations of the marine intertidal snails *Littorina planaxis* and *L. scutulata* in three environments are presented.

2. It is concluded that environmental factors cause the observed size distributions, and the amount of wave action at a given locale appears to be one of the influencing factors. *L. planaxis* was observed to cling to a smooth surface in current velocities of two to three meters per second for 10 seconds.

3. The time for littorines to cycle food completely through the gut varied with size. Snails 0.8 cm. in height required from 2½ to 6 hours.

4. Erosion resulting from the snails' feeding activities was estimated for certain tidepools and found to deepen tidepools one cm. every 16 years. This is of the same order of magnitude as other erosive processes which have been studied in the same region.

5. Growth of *L. planaxis* was found to average 0.02 cm. per month in height increment, and 0.6 mg. per month in dry weight of organic matter.

6. The gross metabolic efficiency was computed and is estimated to be in the neighborhood of 7%.

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THE ECOLOGY OF PHYTOPLANKTON BLOOMS IN MORICHES BAY AND GREAT SOUTH BAY, LONG ISLAND, NEW YORK[1]

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In recent years Great South Bay and Moriches Bay have supported an extremely heavy growth of phytoplankton which characteristically appears early in the spring and persists throughout the summer and fall. At the peak of their growth, the contributing organisms have been found to exceed concentrations of ten million cells per ml., and the resulting turbidity may reduce Secchi disc transparency to less than one foot in places.

Records maintained since 1922 by Mr. J. B. Glancy show that the recurrent annual populations of algae in Great South Bay have consisted almost without exception of small, unicellular, green organisms 2–4 μ in diameter, which have been referred to locally as "small forms." This type of population differs greatly from the mixed communities of diatoms, green flagellates, and dinoflagellates which are typical of the plankton flora in other bays and estuaries of the same general region. Another striking feature is the persistence of the "small form" populations throughout the spring, summer, and fall, in marked contrast to the seasonal succession of dominant species which is commonly observed elsewhere. It is the purpose of this report to analyze the physiological factors which may account for the dominance of "small forms" in the recurrent plankton blooms.

These dense growths of algae have greatly reduced the value of the surrounding region as a recreational area, and are also considered to be the principle cause of the failure of what was formerly a prosperous oyster industry in Great South Bay. Correlated with and suspected as a cause of the algal blooms is the existence of a large duck industry which now consists of over 40 individual farms centered along the tributary streams and coves of Moriches Bay. These farms are so situated that their waste products eventually enter the bays, greatly enriching the water and presumably creating conditions conducive to the development of the plankton blooms.

The results of a study of various aspects of the hydrography, chemistry, and biology of Great South Bay and Moriches Bay, will appear elsewhere (Ryther, unpublished data). This investigation has revealed that the embayments under consideration represent a unique ecological environment which results partly from pollution contributed by the surrounding duck farms, and partly from the topographic and hydrographic features of the area. The pollution not only provides an extensive fertilization of the bay waters with nutrients essential to phytoplankton growth, but, in addition, the presence of organic nitrogen compounds and the low ratio of nitrogen to phosphorus in the pollutants create conditions which differ

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considerably from the chemical composition of natural sea water. The shallow nature of the embayments contributes to the development of abnormally high water temperatures which may reach 30° C. during the summer months. Their extremely low flushing rate results in the retention for long periods of time of both the pollutants and their entraining fresh water, which further builds up the concentrations of nutrients and the resulting phytoplankton crop and also reduces salinities to approximately 50% of normal sea water in the open embayments and less than 10% in the estuaries.

This report includes the results of experiments performed with the two dominant species of "small forms," and, for contrast, with the common neritic diatom, *Nitzschia closterium*. Growth rates of these algae were studied in relation to the temperature, salinity, and nutrient conditions peculiar to Great South Bay and Moriches Bay as described above. The results of these experiments were then related to the distribution of the "small form" population and associated physical and chemical conditions existing in the embayments.

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Culture Methods

The "small form" population of 1952 and 1953 consisted principally of two species of algae. That which was the more numerous was identified by Dr. Wm. Butcher as his recently described *Nannochloris atomus* (Butcher, 1952). The other species was tentatively placed by Dr. Ralph Lewin in the genus *Stichococcus*, possibly *S. cylindricus* Butcher.

Because of their similarity of appearance, it was impossible to distinguish between the two species in routine examinations of the natural population. Consequently they will be considered together as a single community and referred to collectively as the "small form" population.

The *Nannochloris*, *Stichococcus*, and *Nitzschia* used in the experiments were isolated from water samples collected from Great South Bay. Pure cultures were obtained by the agar streak method incorporating the use of penicillin (200 units/ml.) and streptomycin (10–20 units/ml.) in the enriched agar. Colonies isolated from streaks were transferred several times on the antibiotic agar and then inoculated into liquid media.

Pure cultures were used in all experiments involving nutrient studies, while cultures of *Nannochloris* and *Stichococcus* used in the temperature and salinity experiments were unialgal but not bacteria-free.

Growth studies were made with algal cultures grown in 300 ml. of media in 500-ml. Erlenmeyer flasks. Lighting was provided by a bank of mixed daylight and white fluorescent lamps which produced 500 foot candles of illumination.

Temperature was controlled by keeping cultures immersed in running tap water, which provided a range of 20–30° C. over a six-months period by varying the rate of flow of the water. This method permitted control to ± 2° C. for periods of one to two weeks, the normal duration of the experiments. Cultures grown at temperatures of 5–15° C. were maintained to a variability of ± 1° C. in an illuminated, constant temperature box. Except where the effect of temperature upon growth was being studied, cultures were routinely grown at 18–22° C.

The same basic medium was employed for the culture of all three species of algae, and consisted of a modification of the artificial sea water of McClendon *et al.* described in Sverdrup, Johnson and Fleming (1942), enriched with N, P, Si, and Fe. At full strength (34.62‰) it consists of the following, in parts per thousand:

NaCl	26.726	H_3BO_3	0.058
$MgCl_2 \cdot 6H_2O$	2.260	$Na_2SiO_3 \cdot 9H_2O$	0.020
$MgSO_4 \cdot 7H_2O$	3.248	NH_4Cl	0.053
$CaCl_2$	1.153	$Na_2HPO_4 \cdot 12H_2O$	0.020
KCl	0.731	Fe Citrate	0.001
$NaHCO_3$	0.198		
KBr	0.058		

Since the growth of *Nitzschia* is relatively poor in ammonia-nitrogen, as will be demonstrated, NH_4Cl was replaced with 0.10‰ KNO_3 in media used for growing the diatom. Several of the other constituents have been altered in concentration or replaced with other ingredients in the various experiments, as will be discussed below.

Growth in all experimental cultures was determined by cell counts with a Levy hemacytometer. Cultures were grown for periods of 10 days to two weeks, and cell counts were made at intervals of two to three days.

The growth rate, expressed as divisions per day (d), was calculated for the entire period of growth from the expression:

$$d = l_n \frac{c_t}{c_o} \times \frac{1}{t\, l_n 2},$$

where C_t and C_o are cell concentrations at times t and o, respectively.

Experiments and Their Application to Field Observations

1. Nutrients

A. Experiments

A series of laboratory experiments was conducted to determine the relative growth rates of *Nannochloris*, *Stichococcus*, and *Nitzschia* in each of several different forms of nitrogen. Pure cultures of the three species were used, and each was grown at its optimum salinity, as will be discussed in a later section. All three algae were grown in media containing one mg. atom per liter of nitrogen in each of the following forms: nitrate, nitrite, ammonia, urea, uric acid, l-cystine, asparagin, and glycocoll. The resulting growth rates, in divisions per day, are given in Table I.

TABLE I

The effect of the nitrogen source upon the growth rates of Nannochloris, Stichococcus, and Nitzschia

Nitrogen source (1 mg. A N/L)	Growth rate: Divisions per day		
	Nannochloris	*Stichococcus*	*Nitzschia*
NO_3	0.54	0.57	0.37
NO_2	0.65	0.57	0.40
NH_3	0.72	0.61	0.04
Urea	0.62	0.56	0.21
Uric acid	0.68	0.59	0.23
l-cystine	0.77	0.62	0.00
Asparagin	0.68	0.62	0.15
Glycocoll	0.66	0.61	0.07

It may be seen that the diatom grew about equally well in nitrate and nitrite, poorly in ammonia, and slowly or not at all in the organic N compounds. Both *Nannochloris* and *Stichococcus* showed good growth in all of the forms of nitrogen tested. However, the growth rate of both species in nitrite and nitrate was slightly lower than that in ammonia, and, in general, was less than growth rates in the organic compounds. The best growth of *Nannochloris* was observed in cultures containing l-cystine.

TABLE II

The effect of the N:P ratio upon the growth rates of Nannochloris, Stichococcus, and Nitzschia

N:P ratio (by atoms)	Growth rate: Divisions per day		
	Nannochloris	*Stichococcus*	*Nitzschia*
15:1	0.68	0.53	0.39
5:1	1.32	0.88	0.40

In another series of experiments, growth rates of the three species of algae were determined in media containing the same concentrations of nitrate-nitrogen (one mg. atom per liter) but two different concentrations of phosphate-phosphorus (0.066 and 0.200 mg. atoms per liter). By varying the concentrations of phosphorus in this manner, the resulting N : P ratios by atoms in the two media were 15 : 1 and 5 : 1, respectively.

In this experiment, increasing the P concentration, or lowering the N : P ratio, had no effect upon the growth of the diatom, *Nitzschia*, but approximately doubled the growth rates of *Nannochloris* and *Stichococcus* (Table II).

B. Application to field observations

Richards (unpublished data) measured the nitrogen present as uric acid, ammonia, nitrite, and nitrate in the duck farm effluents, the tributaries to Moriches

Bay receiving these effluents, and in Moriches and Great South Bays on August 21, 1952. His analyses showed that uric acid could be detected only in the duck farm effluents, ammonia was present in both the effluents and the tributary streams, while nitrite and nitrate were found nowhere except as traces. No appreciable concentrations of inorganic N in any of the forms tested for was found in either Moriches Bay or Great South Bay.

Phosphorus, on the other hand, appears to have been present in excess of the requirement of the phytoplankton during most of the year, not only in the polluted estuaries, but also throughout the bay waters. Filtered water samples collected from several locations in Great South Bay and Moriches Bay failed to support the growth of *Stichococcus,* if untreated or enriched with phosphate. However, dense growth of the alga occurred in all samples if enriched with ammonia-N indicating that the latter was the principal limiting factor to the growth of the algae population.

The population maxima normally occurred in Moriches Bay and its polluted tributaries. The distribution pattern of the organisms in Great South Bay and Shinnecock Bay is strongly suggestive that their presence in these waters was largely the result of the seaward flushing of Moriches Bay water, and that growth was principally confined to an area close to the source of the nutrient rich duck farm pollutants.

In its original state in the duck wastes, nitrogen occurs as excreted uric acid and amino compounds contained in the undigested food residues. Investigations in this laboratory by Vaccaro, Norton and Plunkett (unpublished) have disclosed that bacteria present in the duck farm effluents are capable of decomposing uric acid with great rapidity. In water samples collected from these effluents, the contained uric acid was found to decrease to 10% of its original concentration in an average of 15 hours at 2° C. The nitrogenous end products of this decomposition were not investigated, but Copeman and Dillman (1937) observed that the decomposition of the uric acid of guano in water was accompanied by an increase in ammonia from 32.2% to 85.0% of the total nitrogen in four days.

According to the classical concept of the nitrogen cycle of the sea, the decomposition of organic nitrogen to ammonia is followed by the nitrification of the ammonia to nitrite and nitrate. This phase of the cycle appears never to occur to any appreciable extent in the Moriches Bay area. Since nitrogen is the limiting factor to the growth of the phytoplankton, it is utilized as quickly as it becomes available and before decomposition to nitrite and nitrate can occur.

It follows that those organisms will have a distinct advantage which are able to utilize the nitrogen in the earliest stages of its decomposition. The laboratory experiments have demonstrated that *Nannochloris* and *Stichococcus* are particularly well adapted to growth in organic nitrogen and its early decomposition products. In contrast, the diatom, *Nitzschia closterium,* grew poorly or indifferently in these N forms. The advantage of the "small forms" over the latter in the Moriches Bay environment is therefore obvious.

Nitrogen and phosphorus are contained in duck faeces at a ratio of approximately 3.3 atoms of nitrogen to one atom of phosphorus. Total N and total P data from the tributaries of Moriches Bay and from the bay itself show N : P ratios ranging from 2.3 : 1 to 4.4 : 1 (Richards, unpublished data). Approximately

one-half of the total phosphorus consisted of inorganic phosphate in these analyses. On the other hand, no appreciable concentrations of available nitrogen were found anywhere in the bay waters, as previously discussed. If it is assumed that half of the total phosphorus and all of the total nitrogen were incorporated in particulate matter, which consisted principally of algal cells, then the N : P ratio of the phytoplankton would range from 4.6 : 1 to 8.8 : 1 by atoms.

Various authors have pointed out that the ratio of nitrogen to phosphorus in open ocean water is rather constant at approximately 15 : 1 by atoms, which is closely reflected in the ratio of these elements in marine phytoplankton (Redfield, 1934; Cooper, 1937, 1938; Fleming, 1940). Harvey (1940) found that natural populations of diatoms utilized about 20 atoms of nitrogen to one atom of phosphorus.

Ketchum and Redfield (1949) showed that laboratory cultures of *Nitzschia closterium* contained N and P at a ratio of 11.6 : 1, but cultures of six species of Chlorophyta, including *Stichococcus bacillaris*, had N : P ratios ranging from 3.5 : 1 to 6.6 : 1, or two to three times as much phosphorus per atom of nitrogen as the diatoms. This compares favorably with the estimated N : P ratio of the phytoplankton of Moriches Bay which consisted predominantly of the Chlorophyta, *Stichococcus* and *Nannochloris*.

These data indicate that there is a basic difference between the chemical composition of the green algae, typical of fresh and brackish water, and oceanic diatoms, at least with respect to the N : P ratio in the cells. The growth studies bear out this contention by demonstrating the fact that the Chlorophyta grow much more rapidly in water containing three times as much phosphorus per atom of nitrogen as normal sea water, while this increase in phosphorus has no effect upon the growth of the diatom, *Nitzschia closterium*.

The N : P ratio of the polluted water of Moriches Bay and its environs thus appears to be another factor favoring the growth of the "small forms" and their competition with organisms similar to *Nitzschia* in their nutritional requirements.

2. *Salinity*

A. Experiments

To determine the effect of salinity upon the growth rates of *Nannochloris*, *Stichococcus*, and *Nitzschia*, each species was grown in a series of dilutions of the artificial medium described above. This series consisted of 100, 75, 50, 25, and 1 per cent solutions of the indicated concentrations of NaCl, $MgCl_2$, $MgSO_4$, $CaCl_2$, and KCl. The concentrations of the other ingredients, which together account for less than $0.5‰$, were not altered to avoid possible deficiencies of those nutrients.

The salinities of the resulting solutions, in ‰, totaled, respectively, 34.51, 25.91, 17.44, 8.91, and 3.80, with an additional $0.048‰$ in each of the *Nitzschia* media as a result of replacing NH_4Cl with KNO_3.

These experiments indicate a salinity optimum at or near that of full sea water for *Nitzschia*, a typical marine species, while the "small forms" grow well within a wide range of salinities, with optima at about 50% sea water, and may be considered as brackish water species (Table III).

TABLE III

The effect of salinity upon the growth rates of Nannochloris, Stichococcus, and Nitzschia

Salinity: °/₀₀	Growth rate: Divisions per day		
	Nannochloris	*Stichococcus*	*Nitzschia*
34.51	0.45	0.44	0.40
25.91	0.61	0.55	0.34
17.44	0.69	0.61	0.28
8.91	0.56	0.58	0.09
3.80	0.37	0.52	0.00

B. Application to field observations

The highest concentrations of "small forms" normally occurred in Moriches Bay in water of approximately $15^0/_{00}$ salinity, or close to the physiological optima of the organisms. While this does not imply that salinity was the factor controlling the distribution of the phytoplankton, particularly in view of the preceding discussion concerning the distribution of nutrients, it nevertheless represents another environmental condition favoring the growth of the "small forms" over that of the more typical marine forms.

Of still greater importance, however, is the fact that *Nannochloris* and *Stichococcus* were able to grow remarkably well within the entire range of salinities tested in the experimental cultures, in contrast to the diatom, which was unable to grow at all in the low salinity cultures. This is particularly significant in view of the wide range of salinities which were observed in Great South Bay and Moriches Bay and their tributaries. Over a million "small forms" per ml. were present throughout the late spring and summer in the upper Forge River, directly opposite a group of duck farms, in water of less than $1^0/_{00}$ salinity. In addition, evidence was obtained in the experiment described in Section 1B that nitrogen-enriched water from Fire Island Inlet ($26^0/_{00}$ salinity) was able to support a prolific growth of *Stichococcus*.

The population which thus becomes established in the highly enriched rivers and estuaries is able to continue growth as it is borne out to sea until its source of nutrients becomes depleted. At no time does salinity limit its growth and thereby permit the succession of other forms.

3. Temperature

A. Experiments

Growth rates of the three algae were determined for cultures grown at temperatures ranging from 5° to 30° C. at intervals of 5° (Table IV). *Nitzschia* was grown in the full strength artificial medium ($34.51^0/_{00}$) while the "small forms" were grown in the half-strength media ($17.44^0/_{00}$) found to give optimum growth in the preceding section.

The diatom multiplied within a temperature range of 5–25° with optimum growth at 15°. Its division rate was relatively high at the lower limit of 5°, but decreased rapidly above 20°.

No appreciable growth of *Nannochloris* or *Stichococcus* occurred at temperatures of 10° or lower, but both species grew rapidly at temperatures of 15–30°. No clear-cut optimum could be detected between 15° and 25°, but growth rates of both "small forms" decreased considerably at 30°.

B. Application to field observations

When field studies were begun on April 1, a mixed diatom bloom occurred in the bays, which consisted of 890,000 *Leptocylindrus minimus*, 35,000 *Thallasiosira nana*, and 12,000 *Skeletonema costatum* per ml. at a station in central Moriches Bay. There were also present at that time 211,000 "small forms" per ml. On May 13 the situation was reversed, with "small forms" dominating the plankton and diatoms reduced to a total of 40,000 cells per ml. The latter subsequently disappeared from the plankton and did not reappear until the following February.

The principle cause of this succession of dominants appears to be temperature. Experimental evidence has shown that *Nannochloris* and *Stichococcus* are unable to grow appreciably at the 10° temperature which prevailed throughout the embayments on April 1, while the diatom, *Nitzschia*, maintained a relatively high growth rate at temperatures as low as 5°.

During the period of May–September, temperatures in Moriches Bay ranged from 13° to 30°. The growth experiments demonstrated that both *Nannochloris* and *Stichococcus* divide rapidly within that temperature range. The seasonal distribution of inorganic phosphorus in the Forge River at a station close to the source of pollution indicates that the heaviest enrichment of the bay waters also occurred during the same period of May–September. The situation therefore exists that during that part of the year when the bay is most heavily enriched with nutrients, temperatures may be expected which will fall approximately within the range for optimum growth of the "small forms."

The month of July, 1952, was characterized by abnormally high temperatures throughout the Long Island area. Water temperatures of the three embayments under consideration ranged from 28–30° during the July 22–23 survey. A

TABLE IV

The effect of temperature upon the growth rates of Nannochloris, Stichococcus, and Nitzschia

Temperature: ° C.	Growth rate: Divisions per day		
	Nannochloris	*Stichococcus*	*Nitzschia*
5	0.00	0.00	0.29
10	0.14	0.12	0.42
15	0.65	0.48	0.48
20	0.80	0.48	0.40
25	0.71	0.43	0.19
30	0.32	0.26	0.00

decrease in the "small form" population at that time was generally observed throughout the area. This may be explained by the fact that the July temperatures exceeded the optima of the "small forms." The division rates of both *Nannochloris* and *Stichococcus* at 30° were observed to be approximately one half of that at 25° (Table IV). This population drop was obviously not associated with a nutrient depletion, as indicated by unusually high inorganic phosphorus concentrations on that date.

The presence of relatively large numbers of "small forms" between October and April, when temperatures were presumably too low to permit their growth, may be explained on the basis of the slow flushing time of Moriches Bay and the low death rate of the organisms. This subject will be discussed at length elsewhere.

It is significant, however, that the slow rate of disappearance of the static population during the winter months not only accounts for the presence of the organisms during that part of the year when they are unable to grow, but it also provides for a substantial seed population by the following spring, when conditions again become favorable for growth. The latter may be one of the chief reasons for the annual recurrence of the "small form" populations.

Discussion

The "small forms," *Nannochloris* and *Stichococcus,* have been found to be particularly well adapted for growth under the peculiar physical and chemical conditions which occur in Great South Bay and Moriches Bay. In addition, it has been brought out that the slow flushing time of the bay waters allows for the retention of a considerable fraction of the summer bloom during the unproductive winter months, thereby providing for a substantial seed population on the following spring. This combination of circumstances alone is perhaps sufficient to account for the presence of "small form" blooms in these waters every year.

It is significant, however, that ecological conditions not only approach an optimum for the growth of the "small forms" but are also quite unsatisfactory for the development of the diatom, *Nitzschia closterium.* It is perhaps suggestive that the physiological characteristics of *Nitzschia* considered here are representative of diatoms, dinoflagellates, and other plankton flora normally found in unpolluted estuaries. While this provides a convenient hypothesis, such an assumption is unwarranted on the basis of the existing evidence.

The utilization of ammonia and amino acids by the green algae has been described by many workers (Schreiber, 1927; Braarud and Føyne, 1930; Algéus, 1946, 1949, 1950, and other papers). If diatoms in general are similar to *Nitzschia* in the matter of their nitrogen utilization, their absence from the summer blooms of Moriches Bay could be explained on that basis alone. This, however, does not appear to be the case.

Harvey (1940) found that natural populations of mixed diatoms could utilize nitrogen as nitrate, ammonia, urea, uric acid, and several amino acids, and that the growth of some species appeared to be better in ammonia than in nitrate. Chu (1943) observed that several diatoms (*Nitzschia palea, Fragilaria crotonensis, Asterionella gracillima*) grew equally well in ammonia or nitrate.

A recent paper by Harvey (1953) describes the exponential growth of *Nitzschia closterium* cultures in media containing ammonia as a source of nitrogen. Since

this is contradictory to the results of the experiments described in this paper, in which *Nitzschia* was found to grow very poorly in ammonia, these experiments were repeated, using both the artificial medium and natural sea water containing one mg. atom per liter of ammonia as a nitrogen source. The growth of *Nitzschia* in both of these media was equally as poor as that reported in the earlier experiments. Since the concentration of ammonia used here was probably higher than that employed by Harvey, there is the possibility that this was the factor which was toxic to the diatom (see Algéus, 1946) although it obviously was not so for either *Nannochloris* or *Stichococcus*. It is equally plausible that the *Nitzschia* used by Harvey and the present author represented different physiological varieties.

From the existing evidence of both the laboratory and the field observations, it appears that the only time of the year when organisms other than the "small forms" are able to dominate the phytoplankton is that period during which temperatures are too low to permit the growth of the Chlorophyta. In this respect, however, the natural situation in the bay waters differs somewhat from that which might be expected from the culture work. The experiments have shown that the "small forms" are able to grow very slowly if at all at the temperatures observed in the bays after October, while diatoms did not begin to flourish there until February. Although the bays were heavily enriched only during the duck growing season, relatively high concentrations of nutrients appear to be present throughout the year, presumably from the decomposition of the rich sediments in the tributaries receiving the duck farm effluents.

A remaining possibility exists that the absence of diatoms and other forms from the water between October and February may have been due, in part, to the production of inhibitory substances by the "small forms" and the accumulation of these products in the bay waters. This is suggested by the work of Pratt and his group (1944, and earlier papers) who found that *Chlorella* produces an antibiotic which inhibits its own growth, and by experiments of Rice (1949) who demonstrated that *Chlorella* and *Nitzschia frustrulum* produce substances which are mutually inhibitory.

Lefèvre and his co-workers (1951) have further shown that filtrates of both laboratory cultures and of pond water containing blooms of different species of algae produce inhibitory effects upon a wide variety of phytoplankton organisms.

It is perhaps significant that Pratt's group (Pratt, Oneto and Pratt, 1945) found that the maximum inhibitory effect of Chlorella was produced by senescent, non-dividing cultures, typical of the late fall and winter population of "small forms" in Great South Bay and Moriches Bay.

Summary

1. The phytoplankton bloom in Great South Bay and Moriches Bay during the spring, summer, and early fall of 1952 consisted of the Chlorophyta, *Nannochloris atomus* and *Stichococcus* sp., to the virtual exclusion of other species. These organisms persisted throughout the year, but were accompanied by minor diatom blooms during the winter and early spring.

2. Growth rates of *Nannochloris, Stichococcus,* and the diatom, *Nitzschia closterium* were determined from laboratory cultures grown under various conditions

of temperature, salinity, and nutrients which are peculiar to the Great South Bay-Moriches Bay area.

3. *Nannochloris* and *Stichococcus* grew well in nitrogen present as nitrate, nitrite, ammonia, urea, uric acid, cystine, asparagin, and glycocoll. *Nitzschia* grew equally well in nitrate and nitrite, but showed poor growth in ammonia and the organic N compounds.

4. *Nannochloris* and *Stichococcus* grew approximately twice as fast in media containing an N : P ratio of 5 : 1 as they did in media with a 15 : 1 ratio of these elements. The growth rate of *Nitzschia* was the same in both media.

5. *Nannochloris* and *Stichococcus* appear to be brackish water forms with salinity optima of about $17^0/_{00}$, but both species grew well within a salinity range of $3–34^0/_{00}$. *Nitzschia*, a typical marine form, was unable to grow in low salinity water.

6. *Nannochloris* and *Stichococcus* grew at temperatures of 10–30° C., with very slight growth at 10° and an optimum range of 15–25°. *Nitzschia* grew within a temperature range of 5–25° with its best growth at 15°.

7. Pollution from duck farms bordering Moriches Bay heavily enriches the bay waters with plant nutrients. The presence of organic nitrogen compounds and the low ratio of nitrogen to phosphorus in the pollutants favor the growth of *Nannochloris* and *Stichococcus* over that of the more typical estuarine phytoplankton.

8. The peculiar nature of the pollutants together with low salinities and high water temperatures occurring at the time and place of maximum enrichment of the bay waters are factors which may explain the persistent dominance of the *Nannochloris-Stichococcus* community in the annually recurring plankton blooms in Great South Bay and Moriches Bay.

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THE PHYSIOLOGY OF INSECT DIAPAUSE. VIII. QUALITATIVE CHANGES IN THE METABOLISM OF THE CECROPIA SILKWORM DURING DIAPAUSE AND DEVELOPMENT[1]

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In the Cecropia silkworm the termination of pupal diapause and the progress of adult development are accompanied by large and predictable changes in respiratory metabolism. Thus, as described in the preceding paper of this series, the respiration of Cecropia midway its adult development is approximately seven times that of the diapausing pupa. In the present study efforts were made to ascertain the enzymatic basis of the quantitative changes in respiration. Considerable evidence was already at hand pointing to pronounced alterations in the cytochrome system in synchrony with the termination of diapause in the eggs of the grasshopper *Melanoplus* (Bodine and Boell, 1934a, 1934b), the eggs of the commercial silkworm *Bombyx* (Wolsky, 1943), and the pupa of the Cecropia silkworm (Sanborn and Williams, 1950; Pappenheimer and Williams, 1952; Schneiderman and Williams, 1952). For this reason attention focussed on the role of the terminal oxidases in relation to diapause and development.

The principal terminal oxidases which have been demonstrated in animals and higher plants are cytochrome oxidase, flavoproteins, and copper-containing proteins such as ascorbic acid oxidase and tyrosinase (Lardy, 1949; Goddard and Meeuse, 1950). Among these, all save ascorbic acid oxidase are thought to function as terminal oxidases in certain animal cells, though the precise role which tyrosinase may play has never been satisfactorily defined (Sussman, 1949).

In animals such as insects, when hemoglobin and other erythrocruorins are absent, carbon monoxide inhibits cytochrome oxidase and tyrosinase (Warburg, 1949), but apparently fails to inhibit flavoproteins or any other enzymes or substrates. It is true that carbon monoxide forms spectroscopically identifiable complexes with certain peroxidases, but peroxidase activity remains uninhibited (Theorell, 1953). Carbon monoxide's inhibition of cytochrome oxidase and tyrosinase can be distinguished in that the former is reversed by light while the latter is not (Warburg and Negelein, 1928; Kubowitz, 1937, 1938). Carbon monoxide therefore affords a remarkably specific tool for tracking the participation of the cytochrome oxidase system in biological reactions. In the present study we have exploited this specificity in an effort to characterize the terminal oxidases of the Cecropia silkworm during diapause and development.

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For reasons considered elsewhere (Schneiderman and Feder, 1954), the effects of high concentrations of carbon monoxide were studied by positive pressure techniques. Animals were placed in transparent, air-filled, polymethyl methacrylate (Lucite) chambers, compressed with carbon monoxide, and measurements of respiration performed by means of respirometers developed for use at high pressures. Under these conditions the oxygen tension remained unchanged at its normal value of one-fifth of an atmosphere, while the carbon monoxide pressure could be increased to as high as seven atmospheres. The positive pressure techniques were supplemented by experiments performed at atmospheric pressure and testing the effects of carbon monoxide, oxygen tension, and cyanide.

Materials and Methods

Diapausing pupae and developing adults of the giant silkworm, *Platysamia cecropia*, were used as experimental animals. In the case of the pupal material the brains were commonly removed to stabilize the animals in permanent diapause (Williams, 1946). In order to avoid the complication of "post-injury metabolism" (Schneiderman and Williams, 1953a), one month or longer was allowed for the recovery of animals subjected to surgical manipulation. All experiments were performed at 25° C.

1. *Metabolic studies*

In studies performed at atmospheric pressure the insects were placed in individual 45-cc. vessels of the type described previously (Schneiderman and Williams, 1953a), and the oxygen consumption determined by the Warburg method. Experiments at positive pressures were carried out in high pressure respirometers (Schneiderman and Feder, 1954). Measurements were begun approximately 1½ hours after compression and continued for 20 to 30 hours in order to compensate for the discontinuous release of carbon dioxide by diapausing pupae (Punt, 1950; Schneiderman and Williams, 1953a, 1953b). The gas volume of the individual respirometers was sufficiently large to preclude any important decrease in oxygen tension during the experimental period.

At the end of positive pressure experiments, acid was added to the alkali and the displaced carbon dioxide measured volumetrically (Schneiderman and Williams, 1953a). The total output of carbon dioxide was measured from the moment the respirometers were sealed to the moment the animals were removed, and the average carbon dioxide production estimated during this period. The over-all respiratory quotient for the duration of the experimental period was calculated from the average carbon dioxide production divided by the average oxygen consumption.

2. *Experimental gases*

The gases were obtained in commercial cylinders and assayed as follows:

Nitrogen (Airco), 99.5% N_2 plus less than 0.5% oxygen. Oxygen (Airco), 99.5% oxygen plus less than 0.5% nitrogen. Carbon monoxide (Matheson Co.), 96.8% carbon monoxide, 0.36% carbon dioxide, 0.97% hydrogen, 1% nitrogen, 0.8% saturated hydrocarbons, 1.19 mg. iron per liter, 0.32 mg. sulfur per liter.

Prior to its use, the carbon monoxide was bubbled through a solution of 10% sodium hydroxide to remove carbon dioxide and iron carbonyl compounds.

In one series of experiments, 200 liters of extremely pure carbon monoxide were prepared by the action of hot concentrated sulfuric acid (C. P. reagent) on formic acid (analytical reagent). The carbon monoxide was passed, in turn, through an aqueous solution containing 5% pyrogallic acid and 25% KOH, a dry ice-acetone trap, $CaCl_2$, $Mg(ClO_4)_2$ ("Anhydrone"), a liquid nitrogen trap, and then compressed to 100 psi in small steel cylinders. Since the effect of this pure carbon monoxide on respiration could not be distinguished from that of the alkali-treated commercial carbon monoxide, the less expensive commercial gas was used in subsequent experiments.

Mixtures of carbon monoxide, oxygen, and nitrogen were prepared under pressure in steel or Lucite tanks and their compositions checked by gas analysis (Scholander and Roughton, 1953).

3. *Cyanide experiments*

To appraise the metabolic effects of cyanide, the insects were first weighed and their water content assumed to equal 75 per cent of the live weight. Then, by means of an extremely small (30) gauge hypodermic needle, each pupa was injected just lateral to the midline of the thoracic tergum with 0.05 to 0.09 ml. of freshly prepared neutralized KCN. The latter's concentration was regulated to establish a specific final concentration after dilution with the fluid volume of the insect. At the pH of the insect, KCN exists almost wholly as HCN; the molar concentration of HCN within the insect was calculated on this basis.

Immediately after injection each pupa was enclosed in a Warburg vessel. As recommended by Robbie (1946), mixtures of KCN and KOH were placed in the vessel for the absorption of carbon dioxide; in this manner the HCN concentration of the chamber was balanced against the internal concentration established within the insect by the injection. At internal HCN concentrations of 10^{-3} *M* or greater, a constant external HCN tension of 5×10^{-4} was employed—the highest concentration that one can establish by means of KCN–KOH mixtures. In one series of experiments the experimental animals were equilibrated *via* the tracheal system with a specific tension of HCN for 60 hours and then studied without the actual injection of cyanide.

Experimental Results

1. *Effects of carbon monoxide at atmospheric pressure on the respiration of diapausing pupae*

In experiments performed on two diapausing pupae and five brainless diapausing pupae the rate of oxygen consumption was first measured in air, then in 6 per cent oxygen plus 94 per cent nitrogen, and, finally, in 6 per cent oxygen plus 94 per cent carbon monoxide (carbon monoxide/oxygen = 16:1). During the course of the experiment the respirometers were flushed periodically with the experimental gases to prevent the uptake of oxygen from appreciably diminishing the oxygen tension in the vessels. As recorded in Table I, it is evident that between the first and eighth hours of exposure to carbon monoxide only one pupa showed any appreciable inhi-

TABLE I

*The effects of carbon monoxide on the oxygen consumption of two diapausing and five brainless diapausing pupae**

Type of pupa	Rate of oxygen consumption (mm.³/gm. live wt./hr.)		Relative rate of oxygen consumption in 16:1 CO/O_2 (%)		
	Air	16:1 N_2/O_2	After 1 hour	Between 1st and 8th hour	Between 8th and 28th hour
Diapausing	9.0 (76%)	11.8 (100%)	126	87	—
Diapausing	11.5 (91%)	12.6 (100%)	132	132	—
Brainless diapausing	8.8 (106%)	8.3 (100%)	116	94	144
Brainless diapausing	9.7 (99%)	9.8 (100%)	85	94	85
Brainless diapausing	9.9 (89%)	11.1 (100%)	95	93	84
Brainless diapausing	11.8 (91%)	13.0 (100%)	106	98	89
Brainless diapausing	13.4 (106%)	12.7 (100%)	73	65	79

* All measurements performed at a total pressure of one atmosphere.

bition of respiration. Between the eighth and twenty-eighth hours four of the brainless diapausing pupae showed about 15 per cent inhibition.

These results demonstrate that only a small fraction of the metabolism of Cecropia pupae is carbon monoxide-sensitive when the carbon monoxide/oxygen ratio is 16:1. To test the effects of still higher ratios, a considerable number of experiments were performed making use of the positive pressure respirometers.

2. *Effects of high pressures of nitrogen on the respiration of brainless diapausing pupae*

Figure 1 illustrates results typical of a number of control experiments in which the respiration of five brainless diapausing pupae was determined in one atmosphere of air and then in air compressed with five atmospheres of nitrogen. It is evident that positive pressures of five atmospheres of an inert gas such as nitrogen were without notable effects on either the rate of oxygen consumption or carbon dioxide output. The slight increase in carbon dioxide output is most probably an artifact attributable to the flushing of stored carbon dioxide from the animal during the period of decompression (Schneiderman and Williams, 1953b).

3. *Effects of high pressures of carbon monoxide on the respiration of brainless diapausing pupae*

Brainless diapausing pupae were placed in air-filled respirometers and their respiration measured after compression with five atmospheres of nitrogen; they were then decompressed to air and the measurements repeated after recompression with five atmospheres of carbon monoxide (carbon monoxide/oxygen ratio of 25:1). Figure 2 records the results obtained in an experiment utilizing five brainless diapausing pupae. A comparison of the respiration in nitrogen and in carbon monoxide reveals that about one-third of the oxygen consumption was inhibited by the high pressure of carbon monoxide. Carbon dioxide production was affected to a lesser degree to yield an apparent increase in the respiratory quotient.

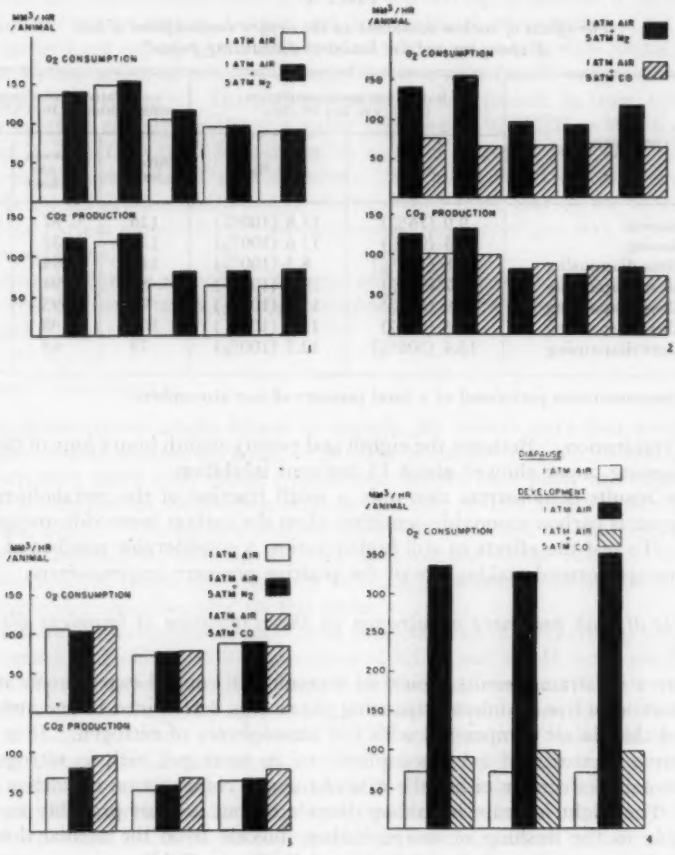

FIGURE 1. The average respiration of five brainless diapausing pupae in air at one atmosphere compared with the average respiration of the same animals in air compressed with 5 atmospheres of nitrogen.

FIGURE 2. The average respiration of five brainless diapausing pupae in air compressed with 5 atmospheres of nitrogen compared with the average respiration of the same animals in air compressed with 5 atmospheres of carbon monoxide.

FIGURE 3. The average respiration of three brainless diapausing pupae, lacking abdominal ganglia, in air at one atmosphere pressure, in air compressed with 5 atmospheres of nitrogen, and in air compressed with 5 atmospheres of carbon monoxide.

FIGURE 4. The average oyxgen consumption of three developing animals on the sixth day of adult development, in air at one atmosphere pressure, and in air compressed with 5 atmospheres of carbon monoxide. The normal average oxygen consumption of diapausing pupae in air at one atmosphere pressure is also recorded.

4. *Effects of high pressures of carbon monoxide on the respiration of brainless diapausing pupae lacking abdominal ganglia*

Taken at face value, experiments of the type just considered suggest that about one-third of the metabolism of diapausing pupae is mediated *via* the cytochrome oxidase system. However, it was noted that diapausing pupae showed a conspicuous depression in the frequency and amplitude of spontaneous muscular movements of the abdominal segments in the presence of high pressures of carbon monoxide. It seemed possible that the observed inhibition by carbon monoxide might arise from a suppression of these movements rather than from inhibition of the pupa as a whole.

This possibility was tested by a repetition of the preceding experiment on a series of brainless diapausing pupae in which the intersegmental muscles of the abdomen had previously been denervated by removal of the chain of eight abdominal ganglia. A total of four such animals were studied in detail. Figure 3 records the respiratory exchange of three of these individuals in air, in air compressed with five atmospheres of nitrogen, and in air compressed with five atmospheres of carbon monoxide. The rates of oxygen consumption under all three conditions were indistinguishable—a result which indicates that in the absence of muscular movements of the abdomen the metabolism of diapausing pupae is insensitive to carbon monoxide.

5. *Effects of carbon monoxide on pilocarpine-stimulated muscular activity and respiration*

It was known from studies to be considered elsewhere that the injection of suitable concentrations of pilocarpine causes diapausing pupae to move their abdomens continuously for up to a year thereafter. Consequently, animals stimulated in this manner afforded ideal material for testing the sensitivity of the abdominal motion and the accompanying respiration to inhibition by carbon monoxide. To this end, each of a series of nine diapausing pupae was injected with 0.1 ml. of 0.1 *M* pilocarpine hydrochloride that had previously been neutralized to pH 6.6 with sodium hydroxide. Two days later, the pupae were enclosed in an air-filled Lucite chamber, compressed with specific pressures of carbon monoxide or oxygen, and the effects on abdominal motion noted.

Carbon monoxide inhibited the abdominal motion to a degree dictated by the carbon monoxide/oxygen ratio. Thus, when a ratio of 10 : 1 was established by the addition of 30 psi carbon monoxide to the initial atmosphere of air, abdominal motion was markedly inhibited. When the ratio was then decreased to 3 : 1 by the addition of 7 psi of oxygen, vigorous movements reappeared. Further compression with carbon monoxide once again restored the inhibition. In virtually all cases abdominal motion ceased when the ratio was as high as 15 : 1, but was resumed within 10 minutes after the decompression and return to air.

Ten days after the experiment just considered the oxygen consumption of five of the continuously wriggling pupae was measured at atmospheric pressure in air, and in specific mixtures of oxygen, nitrogen, and carbon monoxide. The results summarized in Table II reveal that 16 : 1 carbon monoxide/oxygen caused a prompt cessation of the abdominal motion and inhibited the oxygen consumption by approximately 30 per cent.

TABLE II

*The effects of carbon monoxide on the oxygen consumption and abdominal motion of five diapausing pupae injected with 0.1 ml. of 0.1 M pilocarpine hydrochloride**

Rate of oxygen consumption (mm.³/animal/hour)		Relative rate of oxygen consumption in 16:1 CO/O_2 (%)	
Air	16:1 N_2/O_2	During 1st hour	During 3rd hour
81 (89%) (+)	91 (100%) (+)	52 (−)	52 (−)
86 (77%) (+)	112 (100%) (+)	90 (-)	87 (−)
113 (94%) (+)	120 (100%) (+)	58 (−)	58 (−)
133 (116%) (+)	115 (100%) (+)	81 (−)	81 (−)
179 (80%) (+)	223 (100%) (+)	75 (−)	75 (−)
Average: 118 (89%)	132 (100%) (+)	72 (−)	71 (−)

* All measurements performed at a total pressure of one atmosphere.
(+) Records the presence of abdominal motion; (−) the absence of same.

Taken along with the previously mentioned experiments, these findings provide a consistent body of evidence that the contraction of the intersegmental muscles of the diapausing pupa is inhibited by carbon monoxide, whereas the other tissues of the dormant insect are not inhibited by carbon monoxide.

6. *Effects of carbon monoxide on the increased respiration accompanying adult development*

After the termination of pupal diapause the onset and progress of adult development are accompanied by a rapid increase in respiration. Thus on the sixth day of adult development the average respiration is approximately five times that during diapause. In order to ascertain the carbon monoxide-sensitivity of this additional metabolism accompanying development, the respiration of four animals on the sixth day of adult development was first measured in air and then in air compressed with five atmospheres of carbon monoxide. The results, illustrated in the case of the three individuals in Figure 4, demonstrate a striking effect of this 25:1 carbon monoxide/oxygen ratio on the respiration of developing animals. About two thirds

TABLE III

*The effects of carbon monoxide on the oxygen consumption of developing adults**

Days after initiation of adult development	Rate of oxygen consumption (mm.³/gm. live wt./hr.)		Relative rate of oxygen consumption in 16:1 CO/O_2 (%)		
	Air	16:1 N_2/O_2	During 1st hour	During 4th hour	During 8th hour
2	54 (102%)	53 (100%)	68	56	55
5½	84 (129%)	65 (100%)	64	52	39
6½	99 (121%)	82 (100%)	68	56	54

* All experiments performed at a total pressure of one atmosphere.

of the oxygen consumption was inhibited and the metabolism dropped to a level almost as low as that of diapausing pupae. Consequently, it appears that the increased oxygen consumption accompanying adult development is completely or almost completely inhibited by carbon monoxide.

Table III records analogous findings in an experiment in which three developing adults were exposed to a mixture of carbon monoxide and oxygen at a total pressure of one atmosphere. To compensate for the utilization of oxygen, the Warburg vessels were reflushed with the experimental gas every 2½ hours. It will be noted that the 16:1 carbon monoxide/oxygen inhibited the oxygen consumption of the developing insects by approximately 50 per cent.

7. *The effects of carbon monoxide on the post-injury metabolism of diapausing pupae*

In addition to the increased metabolism which accompanies the onset of adult development, the pupa during diapause can undergo a substantial increase in its metabolism under certain experimental conditions. Thus, after small localized injury to the pupal integument, the oxygen consumption and carbon dioxide production are considerably enhanced for one to several weeks thereafter (Sussman, 1952; Schneiderman and Williams, 1953a). This result has been regularly observed in both normal pupae and in pupae immobilized by prior removal of the abdominal ganglia.

Experiments were performed to test the sensitivity of the injury metabolism to carbon monoxide. To this end, the brains and abdominal ganglia were removed from six diapausing pupae. Two months later the rate of oxygen consumption was determined for each animal. A V-shaped 4-mm. incision was then made in the thoracic tergum of each animal, and the rate of oxygen consumption measured one day later. Three of the pupae were then placed in air-filled respirometers, compressed with five atmospheres of carbon monoxide, and the measurements repeated.

TABLE IV

The effects of carbon monoxide on the injury-stimulated respiration of brainless diapausing pupae lacking abdominal ganglia and connectives

		Rate of oxygen consumption* (%)					
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Prior to injury	In air	100	100	100	100	100	100
24 hours after injury	In air	158	208	108	198	146	146
30 hours after injury	In air	153	183	118	—	—	—
	In CO	—	—	—	183	152	128
Percentage difference between 1st and 2nd post-injury measurements		−3	−12	+9	−8	+4	−12

* Initial pre-injury oxygen consumption varied from 91 to 143 mm.³/animal/hour.

The respiration of the other three pupae was again measured in air at one atmosphere. The results recorded in Table IV show that the extra respiration stimulated by injury is *uninhibited* by carbon monoxide.

8. *Effects of cyanide on the respiration of diapausing pupae*

Diapausing pupae were injected with specific concentrations of cyanide and their oxygen uptake then ascertained. A typical set of measurements is plotted in Figure 5. In control experiments in which distilled water was injected, the oxygen uptake began to increase about five hours after the injection, and the typical pattern of injury metabolism became apparent. When cyanide was injected

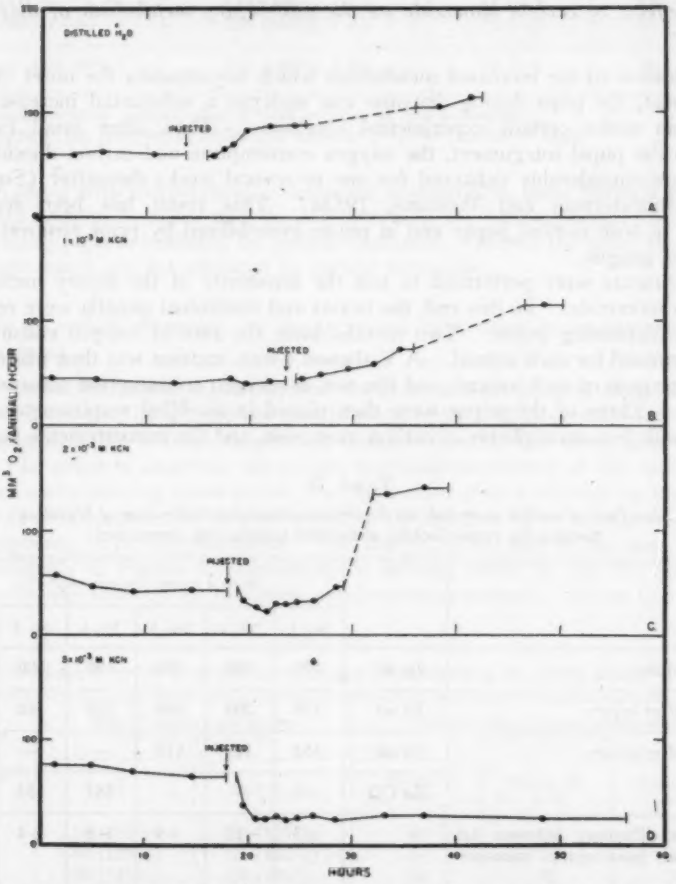

FIGURE 5. The effects of cyanide injection on the oxygen consumption of diapausing pupae. Concentrations of cyanide refer to calculated final internal concentrations.

to attain a final internal concentration of 10^{-3} *M*, a slight inhibition was usually observed, though in some cases the effect did not differ from that of distilled water. With further increase in cyanide to a final concentration of 2×10^{-3} *M*, an immediate inhibition of 50 per cent always occurred which persisted at a steady level for about ten hours and then gradually returned to normal. Still higher concentrations of cyanide (5×10^{-3} *M* and above) caused a prompt inhibition of 70 to 90 per cent, followed by the death of the animal several days later.

In the interpretation of experiments of this type it is necessary to separate the effects of injury from those of cyanide. This can most easily be accomplished by

TABLE V

The effects of cyanide on the respiration of diapausing pupae

Calculated internal concentration of HCN	Prior to injection		After injection	
	Average rate of oxygen consumption (mm.³/gm. live wt./hr.)	Lowest hourly rate of oxygen consumption as per cent of average initial rate*	Lowest hourly rate of oxygen consumption in 48-hour period as per cent of average initial rate	Behavior
0	10.7	97	98	Normal
	11.4	87	96	
	15.8	79	104	
	17.4	92	108	
	Average	89	102	
5×10^{-4} *M*	9.9	92	80	Normal
	10.3	96	102	
	10.5	88	97	
	12.1	91	89	
	12.5	94	98	
	Average	92	93	
5×10^{-4} *M* (Exposed to HCN gas for 60 hours but not injected)	8.1	95	93	Normal
	9.1	95	91	
	11.0	91	98	
	13.0	97	87	
	13.5	90	96	
	14.7	93	76	
	Average	93	90	
1×10^{-3} *M*	8.5	87	110	Normal, or slight decrease in abdominal motility
	9.7	95	107	
	11.4	88	94	
	11.4	94	51	Conspicuous decrease in abdominal motility for 12 to 24 hours
	10.5	83	85	
	12.2	100	72	
	28	93	67	
	Average	91	84	

* Measured during a four- to eight-hour period prior to cyanide injection.

TABLE V (*Continued*)

Calculated internal concentration of HCN	Prior to injection		After injection	
	Average rate of oxygen consumption (mm.³/gm. live wt./hr.)	Lowest hourly rate of oxygen consumption as per cent of average initial rate*	Lowest hourly rate of oxygen consumption in 48 hour period as per cent of average initial rate	Behavior
2×10^{-3} *M*	8.1 11.0 13.0	95 91 97	53 50 52	Electrically inexcitable for one day. Spontaneous abdominal movements reappeared after 2 days
	Average	94	52	
5×10^{-3} *M*	9.1 13.5 14.7	96 90 93	39 13 36	Died
	Average	93	29	
1×10^{-2} *M*	11.9 13.7 20.2	90 97 93	21 19 26	Died
	Average	93	22	

focusing attention on the oxygen uptake in the first few hours after injection and prior to the onset of the injury metabolism. As a measure of cyanide inhibition we chose the *lowest oxygen uptake* measured over a one hour interval during this period. To prevent any normal hour-to-hour variations in oxygen uptake from being interpreted as cyanide inhibition, we also recorded the *lowest hourly rate* of oxygen consumption during a four- to eight-hour period prior to cyanide injection. Table V summarizes such calculations on a series of 31 diapausing pupae. Detectable inhibition of abdominal motion and of respiration appeared only when the internal cyanide concentration, as calculated, was increased to or above 10^{-3} *M*.

In the evaluation of these findings it is worth recalling a technical difficulty mentioned in the section on Methods; namely, that it was impossible by the use of KCN–KOH mixtures to establish HCN concentrations higher than 5×10^{-4} *M* in the air surrounding the insect. Consequently, at internal concentrations higher than 5×10^{-4} *M*, detoxification and unspecific combinations of the injected HCN, along with the loss of HCN by diffusion from the tracheal system, necessarily reduced the internal concentrations below the calculated values. This fact complicated a decision as to the cyanide-sensitivity of the abdominal muscles. However, in experiments of short duration performed on a total of 18 diapausing pupae, we found that 10^{-3} *M* cyanide uniformly impaired the contractility of the abdominal muscles of normal pupae and totally eliminated the contractility of pilocarpine-stimulated muscles. In the latter case the muscles no longer responded to electrical stimulation. Consequently, we conclude that the carbon monoxide-sensitive abdominal muscles of diapausing pupae are likewise cyanide-sensitive.

Warburg (1949) emphasizes the fact that cyanide is a specific inhibitor of heavy metal enzymes only at concentrations up to about 10^{-3} *M*; above this level cyanide undergoes significant combinations with a large number of substrates and metabolic intermediates. In the case of Cecropia we attribute the lethal effects of cyanide concentration of 5×10^{-3} *M* and above to these unspecific side-reactions of cyanide.

9. *Effects of cyanide on the respiration of developing adults*

After the termination of diapause and the initiation of adult development, the effect of cyanide was easier to ascertain by virtue of the absence of injury metabolism, the latter being peculiar to diapausing pupae (Schneiderman and Williams, 1953c). Table VI summarizes the inhibition by cyanide of the respiration of 19

TABLE VI

The effects of cyanide on the respiration of developing adults

Calculated internal concentration of HCN	Days after initiation of adult development	Average rate of oxygen consumption prior to injection (mm.³/gm. live wt./hr.)	Oxygen consumption as per cent of average initial oxygen consumption at intervals after injection					Behavior after injection
			1.5 hrs.	8.5 hrs.	20 hrs.	26 hrs.	40 hrs.	
10^{-4} *M*	3	69	(124)	(66)			(110)	Emerged
	9	119	79	52			122	Emerged
	12	136	83	41			124	Emerged
	15	166	70	39			54	Died
	17	279	(92)	(20)			(30)	Died
		Average	77	44			100	
2×10^{-4} *M*	10	112	90		5	5		Died
	12	114	68		7	7		Died
	12	140	67		6	6		Died
	13	186	63		5	5		Died
	14	206	69		12	12		Died
		Average	71		7	7		
5×10^{-4} *M*	9	95	57		4		6	Died
	10	100	52		6		7	Died
	10	118	43		25		6	Died
	11	128	41		10		7	Died
	13	160	42		12		7	Died
		Average	47		11		7	
10^{-3} *M*	8	110	27		6		7	Died
	11	150	21		7		6	Died
	12	131	30		9		7	Died
	13	162	23		9		7	Died
		Average	25		8		7	

Figures in parenthesis () were not included in the average since these animals were not at comparable stages of adult development.

developing adults. The results have been averaged for animals between the 8th to 15th day of adult development. In contrast to the findings on diapausing pupae, cyanide at final concentrations of 2×10^{-4} *M* or higher was lethal; moreover 10^{-4} *M* cyanide now inhibited the oxygen uptake 35 to 80 per cent. In Table VI it also appears that the proportion of total metabolism which was cyanide-sensitive undergoes definite increase during the course of the twenty-two day period of adult development.

10. *Effects of oxygen tension on the respiration of brainless diapausing pupae lacking abdominal ganglia*

The experimental results, up to this point, demonstrate that systematic changes occur in the insect's dependency on metabolism sensitive to carbon monoxide and to cyanide. Aside from the intersegmental muscles of the abdomen, the metabolism of the diapausing pupa and the extra metabolism which it exhibits in response to injury are substantially insensitive to carbon monoxide and cyanide; by contrast, the metabolism of the developing insect is markedly inhibited by these agents.

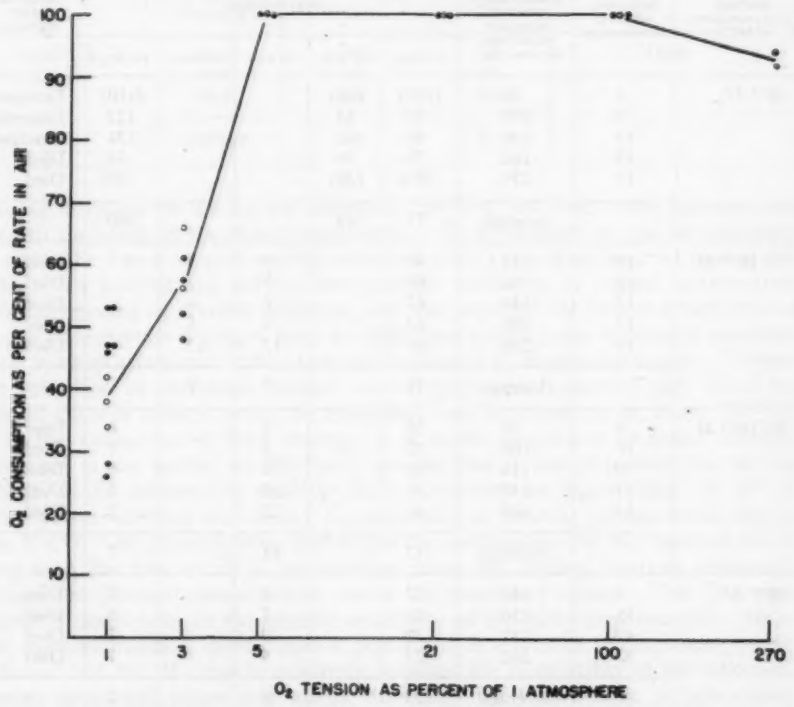

FIGURE 6. The effects of oxygen tension on the oxygen consumption of three brainless diapausing pupae lacking abdominal ganglia and connectives. The abscissa is marked off in arbitrary units.

Carbon monoxide-stable respiration has generally been found to require oxygen tensions far higher than does carbon monoxide-sensitive respiration (see Discussion). For this reason the effect of oxygen tension was studied in relation to the metabolism of diapause and development.

The oxygen consumption of three brainless diapausing pupae, previously immobilized by the removal of their abdominal ganglia, was determined at a series of oxygen tensions ranging from 1 to 270 per cent of an atmosphere of oxygen. The results of measurements at six different oxygen tensions are recorded in Figure 6. Each individual determination represents a steady-state value obtained after exposure to the gas mixtures for several hours. After each determination the pupae were returned to air for three days. The respiration in air was then re-determined before the pupae were exposed to a new oxygen tension.

As Figure 6 indicates, the oxygen consumption was independent of oxygen pressure at tensions from 5 per cent to 100 per cent of an atmosphere. In 3 per cent oxygen a conspicuous decrease was evident. But even in one per cent oxygen the average oxygen consumption was still 40 per cent of that in air. At the extremely high oxygen tension of 2.7 atmospheres, there appeared to be a slight depression, presumably attributable to the toxic effect of oxygen (Williams and Beecher, 1944).

Four pupae were stored in one per cent oxygen for ten hours. When returned to air, the oxygen consumption increased approximately 25 per cent above the normal rate in air and persisted at this level for several hours. This observation suggests the accumulation of a small but definite oxygen debt at the low oxygen tension. It is clear, however, that diapausing pupae possess limited ability to accumulate an oxygen debt for, as demonstrated in numerous experiments, several days of exposure to 0.5 per cent oxygen is lethal (L. D. 50% = 3 days at 25° C.).

Discussion

1. *Insensitivity of insects to compression*

Interpretation of the experimental results obviously requires a decision as to whether the experiments at high gaseous pressures were complicated by unspecific or narcotic effects of pressure *per se* (Behnke, 1940; Lawrence *et al.*, 1946).

As far as nitrogen is concerned, narcotic effects have not been demonstrated in any experiments on insects. Chadwick and Williams (1949) found that *Drosophila* could fly in 4.5 atmospheres of nitrogen plus one atmosphere of air, although the wingbeat frequency was decreased because of the increased gas density. Case and Haldane (1941) observed that *Drosophila* was active at 10 atmospheres of nitrogen plus one atmosphere of air, and Williams (unpublished) found that seven hours of exposure to 24 atmospheres of nitrogen plus one atmosphere of air failed to affect the vitality of *Drosophila* upon subsequent return to air. Moreover, experiments on the Cecropia silkworm at all stages of development, from egg to adult, have demonstrated that prolonged exposure to 6.7 atmospheres of nitrogen plus one atmosphere of air fails to retard embryonic or adult development, heart beat, movement, or the spinning of the cocoon (unpublished observations). And as demonstrated in the present study, the oxygen consumption was the same in air and in air compressed with five atmospheres of nitrogen. These results give assurance that nitrogen, at the pressures utilized, was not a narcotic.

We feel that the same conclusion is valid in the case of carbon monoxide. Thus, the oxygen consumption of immobilized diapausing pupae was the same in air and in air compressed with five atmospheres of carbon monoxide. Moreover, we shall subsequently show that many of the effects of high pressures of carbon monoxide on the post-diapausing insect are reversed by light (Schneiderman and Williams, 1954). Consequently, high pressure techniques appear to be useful and uncomplicated tools for experiments on insects.

2. Significance of carbon monoxide-sensitive respiration

As mentioned in the Introduction, suitable pressures of carbon monoxide are known specifically to inhibit the function of two enzymes, cytochrome oxidase and tyrosinase. Inhibition of cytochrome oxidase is reversed by light; inhibition of tyrosinase is not. Though the light-reversibility of carbon monoxide's action on post-diapausing Cecropia has already been described in the case of the male sex cells of Cecropia (Schneiderman, Ketchel and Williams, 1953) and will be considered in further detail at a later occasion, for our present purposes the phenomenon is doubly significant since it excludes tyrosinase as the critical target of carbon monoxide. This conclusion is further substantiated by the failure of phenylthiourea or any of a number of other potent anti-tyrosinases to duplicate the effects of carbon monoxide or of cyanide (Schneiderman and Williams, 1953a). We are therefore persuaded that the actions of carbon monoxide on Cecropia are due to its combination with cytochrome oxidase.

The factors which condition the quantitative effects of carbon monoxide on respiration mediated by the cytochrome oxidase system are four in number: (1) the relative affinity of the insect's cytochrome oxidase for carbon monoxide and for oxygen; (2) the carbon monoxide/oxygen ratio established at the site of enzyme action; (3) the degree to which cytochrome oxidase limits the transfer of electrons from substrate to oxygen; and (4) the oxidation of carbon monoxide to carbon dioxide. We shall briefly consider each of these points as it pertains to the present study.

Detailed measurements of the relative affinity of cytochrome oxidase for carbon monoxide and oxygen are available only in the case of yeast (Warburg, 1949) and mammalian heart muscle (Ball *et al.*, 1951). A 17:1 carbon monoxide/oxygen ratio inhibits the cytochrome-catalyzed respiration of yeast 75 per cent and the cytochrome oxidase activity of heart muscle 64 per cent. Since the relative affinities are so similar for such dissimilar cell types, it is a reasonable presumption that the insect cytochrome oxidase does not differ greatly. This probability has been confirmed by the finding that a carbon monoxide/oxygen ratio of 16:1 causes a light-reversible inhibition of the cytochrome oxidase activity of homogenates of the thoracic muscles of Cecropia moths by approximately 50 per cent (Pappenheimer and Schneiderman, unpublished observations).

In the positive pressure experiments on Cecropia a ratio of 25:1 was routinely established in the air surrounding the insect. The utilization of oxygen in the respirometer gradually lowered the oxygen tension over a period of 30 hours from 21 per cent to as low as 14 per cent, and thus increased the carbon monoxide/oxygen ratio. And, in each instance, the utilization of oxygen in the tissues decreased the internal oxygen tension still further. Consequently, the recorded ratio of 25:1 is

a minimal estimate of the effective carbon monoxide/oxygen ratio that was actually established in the insect's tissues at the site of enzyme action. For these several reasons we conclude that carbon monoxide in the positive pressure experiments effectively inhibited a large proportion of the insect's cytochrome oxidase—probably not far short of 100 per cent.

As mentioned above, one might anticipate that an excess of cytochrome oxidase relative to cytochrome *c* and other electron donors would tend to camouflage the participation of cytochrome oxidase in the metabolism of diapause. However, it is worth recalling that carbon monoxide combines exclusively with reduced cytochrome oxidase; that is, with functional oxidase receiving electrons from cytochrome *c*. An excess of the oxidase would necessarily be present in the oxidized form and therefore incapable of combining with carbon monoxide.

Finally, there is circumstantial evidence that the oxidation of carbon monoxide to carbon dioxide was not a complicating factor in the present study. If such an oxidation occurred, as described in the case of frog muscle by Fenn and Cobb (1932) and Stannard (1940), the oxidation of each molecule of carbon monoxide would be recorded manometrically as 1½ molecules of oxygen consumed and one molecule of carbon dioxide produced. The theoretical R.Q. of this process is 0.66, and in the case of the Cecropia pupa would thus tend to *decrease* the normal R.Q. of 0.78. However, since as recorded in Figures 2 and 3, a slight *increase* in R.Q. was actually observed in the presence of carbon monoxide, the oxidation of carbon monoxide was not a serious complication in the present experiments.

Thus, in summary, the conclusion seems acceptable that metabolism insensitive to high ratios of carbon monoxide/oxygen signals the utilization of terminal oxidases other than cytochrome oxidase.

3. *Significance of cyanide-insensitive respiration*

Cyanide is a far less specific inhibitor of cytochrome oxidase than is carbon monoxide. It inhibits not only cytochrome oxidase, but also catalase, peroxidase, and tyrosinase, and, as previously mentioned, at concentrations higher than about 10^{-3} *M*, cyanide also combines with various substrates, metabolic intermediates, and enzymes possessing carbonyl groups. For this reason cyanide-sensitivity is far less significant than cyanide-insensitivity. Cyanide-insensitivity strongly suggests that neither cytochrome oxidase nor any of a number of other enzymes are prerequisite for the reaction in question.

4. *Respiration during diapause and development*

As judged by its insensitivity to cyanide and carbon monoxide, virtually all of the metabolism of the diapausing pupa appears to proceed *via* pathways independent of cytochrome oxidase, save for the metabolic events responsible for the contraction of the abdominal muscles. It is therefore of particular interest and importance that the intersegmental muscle of the abdomen is the only major tissue within the diapausing pupa containing a high concentration of the classical cytochrome system (Williams, 1951; Pappenheimer and Williams, 1952).

The termination of diapause and the onset of adult development, however, usher in a new situation in which a progressively larger fraction of metabolism becomes dependent on the presence of a functional cytochrome oxidase system. Evidently,

at this time, a carbon monoxide- and cyanide-sensitive respiration mediated by cytochrome oxidase is superimposed on the carbon monoxide- and cyanide-stable metabolism of diapause.

Analogous changes in the relative activities of carbon monoxide-sensitive and carbon monoxide-stable respiratory systems have been demonstrated in a variety of plants, animals, and micro-organisms (Bodine and Boell, 1934a, 1934b; Wolsky, 1943, 1949; Paul, 1951). In the case of Cecropia the shift to cytochrome oxidase-mediated respiration is synchronized with the action of the insect's "growth and differentiation hormone" in terminating the pupal diapause—a correlation which suggests that the change in metabolism, in itself, is a part of the biochemical action of the hormone.

5. *Effects of oxygen tension on the respiration of brainless diapausing pupae*

The experimental results demonstrate that the metabolism of diapausing Cecropia pupae becomes independent of oxygen tension when the latter is five per cent of an atmosphere or higher. The tension of oxygen is usually considered to limit respiration at the cellular level only when it approximates zero (Krogh, 1916; Oppenheimer, 1925); this consideration is valid in most organisms since cytochrome oxidase, the usual terminal oxidase, is saturated by oxygen at tensions ranging from 0.25 to 2.5 mm. Hg (Winzler, 1941). However, flavoproteins when functioning as terminal oxidases are ordinarily thought to be unsaturated at low oxygen tensions. Thus, the "old yellow enzyme" which Warburg and Christian (1932) isolated from yeast was markedly influenced by variations in oxygen tension: in 100 per cent of an atmosphere of oxygen the respiration which it mediated was nearly five times that in 5 per cent oxygen. A corresponding dependency on oxygen tension has also been observed for flavoprotein-mediated respiration *in vivo*. Thus, in thin sections of the *Arum* spadix (James and Beevers, 1950), flavin-catalyzed oxygen uptake increased progressively as the oxygen tension was raised to one atmosphere.

The fact that the respiration of diapausing pupae is independent of oxygen at tensions greater than 5 per cent of an atmosphere, and the further fact that one per cent oxygen sustains 40 per cent of the normal respiration suggest that the carbon monoxide- and cyanide-stable oxidase of Cecropia differs from the flavoproteins reported in plants and bacteria and studied *in vitro*. However, we cannot exclude the possibility that such an oxidase may be present in relative excess in Cecropia and that it may fail to limit electron transmission even when driven slowly at low oxygen tensions.

6. *Identification of the terminal oxidases mediating respiration in diapausing pupae*

The results of the present study permit the following characterization of the terminal oxidases in diapausing Cecropia pupae. The principal terminal oxidase in the intersegmental muscles of the abdomen is cytochrome oxidase; in other major tissues of the diapausing insect it is not cytochrome oxidase. The latter unknown oxidase is uninhibited by high concentrations of carbon monoxide (carbon monoxide /oxygen ratios of 25: 1), or by cyanide concentrations up to 10^{-3} *M*, or by phenylthiourea. Moreover, the oxidase in question is saturated by oxygen tensions of 5 per cent of an atmosphere or less.

On the basis of these several lines of evidence, the most probable candidates appear to be either an autoxidizable flavoprotein transferring electrons from reduced pyridine nucleotides to molecular oxygen, or an autoxidizable heme-containing enzyme which fails to combine with either cyanide or carbon monoxide.

Summary

1. The respiration of the Cecropia silkworm was studied after the injection of cyanide or in the presence of specific mixtures of oxygen, nitrogen, and carbon monoxide. Positive pressure techniques were utilized to test the effects of carbon monoxide/oxygen ratios as high as 25 : 1.

2. It was found that the respiration of the diapausing pupa is only slightly affected by high concentrations of carbon monoxide or cyanide. This minor effect was accounted for in terms of the cyanide- and carbon monoxide-sensitivity of the contraction of the intersegmental muscles of the pupal abdomen. The other tissues in the dormant insect showed no detectable inhibition by high concentrations of cyanide or carbon monoxide.

3. The termination of the pupal diapause and the progress of adult development are accompanied by a marked increase in the insect's sensitivity to cyanide and carbon monoxide. The effects of these agents are then no longer limited to muscular tissue but extend to the insect as a whole. Cyanide or carbon monoxide appear to act exclusively on the extra metabolism accompanying development and, thereby, to reduce the overall metabolism to the old diapausing level.

4. The modes of action of cyanide and carbon monoxide within the diapausing and non-diapausing insects are considered in detail. Insensitivity to these agents, as in most tissues of the diapausing pupa, argues in favor of the presence and utilization of a terminal oxidase other than cytochrome oxidase.

5. It is concluded that cytochrome oxidase is the principal terminal oxidase of only the somatic musculature of the diapausing pupa. Months later, with the termination of the pupal diapause, cytochrome oxidase becomes the principal terminal oxidase of the growing, post-diapausing insect as a whole.

6. These qualitative changes in the insect's metabolism are synchronized with the secretion of the hormone responsible for the termination of diapause and the development which follows, and appear to be a more or less immediate result of the hormonal action.

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A RESPIROMETER FOR METABOLIC STUDIES AT HIGH GASEOUS PRESSURES[1]

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The use of carbon monoxide as a specific inhibitor of cytochrome oxidase is accompanied by one serious complication—the inhibition is reversed by oxygen (Warburg, 1949). The degree to which one can inhibit the enzyme is therefore dependent, not alone on the carbon monoxide pressure, but also on the pressure of oxygen that is simultaneously present. In short, the inhibition of cytochrome oxidase is a function of the carbon monoxide/oxygen ratio. To achieve an effective inhibition of the enzyme, this ratio must be high. Fifty per cent inhibition requires approximately a 10 to 1 ratio; 75 per cent inhibition, a 20 to 1 ratio. For greater degrees of inhibition, still higher ratios are necessary (Warburg, 1949; Ball *et al.*, 1951).

To achieve this goal it has been customary to use an atmosphere containing 95 per cent carbon monoxide and 5 per cent oxygen; indeed, an atmosphere of 98 per cent carbon monoxide and 2 per cent oxygen has occasionally been employed. However, oxygen at these low pressures fails to satisfy the normal respiratory requirements of most plants and animals *in vivo* or *in vitro* (Tang, 1933). Experimental results are thereby complicated by anoxia, and there is uncertainty as to whether an observed effect is due to the presence of carbon monoxide or a deficiency in oxygen. The earth's atmospheric pressure (760 mm. Hg) is too low to permit one to inhibit cytochrome oxidase effectively by substituting carbon monoxide for the nitrogen in air. If the oxygen tension is to be maintained at its normal value (⅕th atmosphere), then several atmospheres of carbon monoxide must be superimposed.

For this reason there has long been a need for a simple and practical method for measuring oxygen consumption and carbon dioxide production at high gaseous pressures. This objective seems doubly attractive in view of the insensitivity of most biological preparations to pressure *per se*, as long as the latter is not extremely high.

Two methods have been described for this purpose; namely, that of Libbrecht and Massart (1937) and that of Stadie and Riggs (1944). Both of these methods utilized a pressure chamber containing a manometric apparatus of the Warburg type. The chamber designed by Stadie and Riggs had a capacity of 60 liters and enclosed 6 Warburg manometers and vessels. The apparatus was con-

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structed so that the necessary manipulations and readings were made from the outside, while high pressure and constant temperature were maintained inside. As might be anticipated, such an apparatus was extremely costly, presented an explosive hazard, and required careful checks to police a dozen separate gaskets and fittings.

The present paper describes a simple and practical technique for measuring gas exchange at positive pressures up to seven atmospheres. The respirometer itself is inexpensive, safe, leak-proof, and yields results of the same degree of accuracy as conventional manometric and volumetric techniques. In its present form it is suitable for studies of intact animals, plants, and tissues where agitation is not required. However, the principle of the technique is readily adaptable to studies of solutions, slices, and homogenates, and also to studies at pressures below atmospheric.

Principle of Method

A small glass respiration chamber, containing the experimental animal plus a carbon dioxide absorbant, is joined to a graduated capillary tube. Provisions are made so that the capillary can subsequently be sealed with a fluid index drop. After assembly, the respiration chamber is enclosed in a large polymethyl methacrylate (Lucite) chamber capable of withstanding high internal gas pressure. At the start of the experiment the capillary tube is patent; the lumen of the capillary therefore affords a direct connection between the gas space of the respiration chamber and the Lucite compensation chamber. Consequently, when the latter is filled with gas to a desired pressure, gas passes through the capillary and fills the respiration chamber at the same pressure. When a desired pressure is attained, an index drop is tipped into the graduated capillary, thus sealing the respiration chamber. The measurements are then performed in the same manner as in an ordinary volumeter of the Fenn type (Fenn, 1935). Since the plastic compensation chamber is closed off from the outside air, excursions of the index drop are independent of changes in atmospheric pressure (*cf.* Gerard and Hartline, 1934).

Apparatus (see Fig. 1)

a. Compensation chamber. The plastic compensation chamber is a transparent Lucite cylinder fitted with brass endplates, gaskets, and needle valves. Figure 2 shows the chamber and its component parts. The Lucite cylinder is 4″ I. D. × 4.50″ O. D. × 18″ long. The endplates are 5″ × 5″ × 0.5″ brass plates with a 0.25″ deep circular channel milled on the inner surface to receive the Lucite cylinder. A rubber gasket is inserted into this channel. Half-inch Hoke needle valves are threaded and silver brazed in the center of each endplate. The two endplates are held together by four brass rods, 0.5″ in diameter. Endplate A is bolted to the rods, while endplate B is removable. On endplate A the rods extend 0.5″ beyond the reducing valve so that the tank may be placed on end. The internal volume of the assembled compensation chamber was 3460 cc.

b. Capillary volumeter. The size of the respiration chambers and capillaries is dictated by the dimensions of the experimental animal, the rate of oxygen consumption, and the desired sensitivity. The size most frequently used in this laboratory

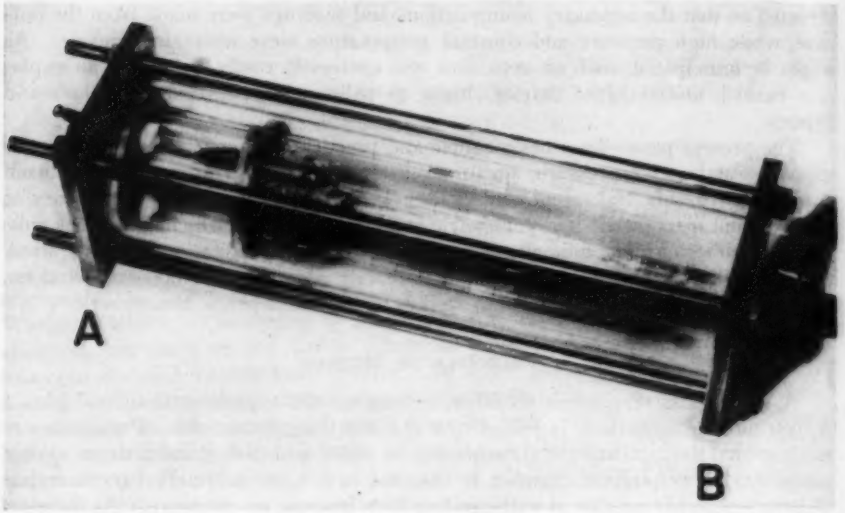

FIGURE 1. High pressure respirometer: an animal-containing *capillary volumeter*, a *reference volumeter*, and a *capillary barometer* are mounted on the *capillary volumeter frame*.

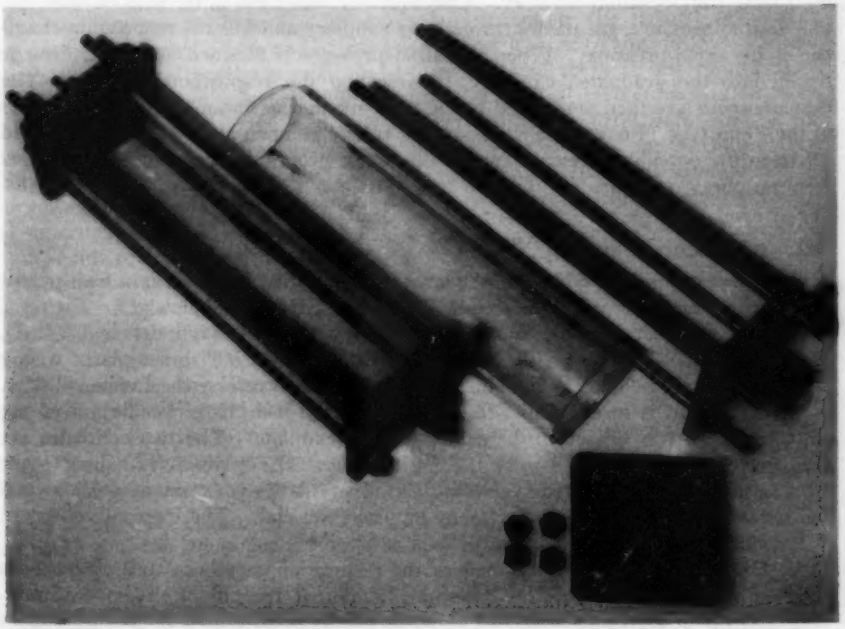

FIGURE 2. A 3460-cc. Lucite compensation chamber and its component parts.

is shown in Figure 3.[4] It consists of a 20-cm. length of 2 mm. bore capillary tubing, calibrated in 0.005 cc. units, and fitted by a 20/40 standard taper joint to a 30-cc. Pyrex shell vial, 85 mm. long. To the tip of the capillary is fused a 3.5 cm. length of 7-mm. bore Pyrex tubing. The latter is slightly constricted at its distal open end and serves as a reservoir for the index drop solution. The calibrated volume of each capillary is 550 mm.[3] The total volume of each volumeter to the tip of the capillary is 45 cc.

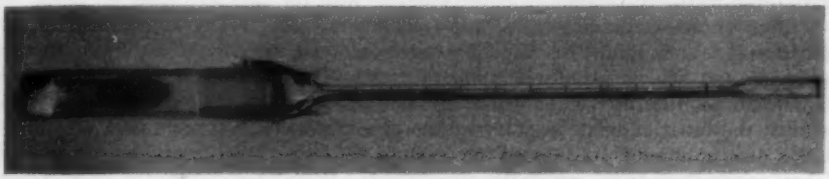

FIGURE 3. A 45-cc. capillary volumeter.

The respiration chamber can be attached to a standard Warburg manometer by means of an adapter previously described (Schneiderman and Williams, 1953). Thus the respiration of the animal in air before and after exposure to high pressure may be conveniently measured by conventional methods without removal of the animal from the respiration chamber.

c. Capillary barometer. Measurements of absolute pressure, accurate to within one per cent, are required for the proper determination of gas exchange in the present apparatus (see discussion of calculations below). Since the standard Bourdon type pressure gauges are subject to errors in excess of 5 per cent, a capillary barometer is utilized. Each such barometer consists of a one-cc. pipette of approximately 2 mm. internal diameter, graduated in 0.01 cc., and sealed at one end. By means of a long hypodermic needle a drop of colored detergent solution is placed in the closed end of the capillary and another drop at the beginning of the graduations. In each experiment three capillary barometers of this type are enclosed in the Lucite compensation chamber. The pressure in the closed system is calculated by application of the gas law from readings of the capillary barometer taken before and after compression and from a reading of the local barometric pressure.

d. Water bath. Glass aquaria make the most satisfactory water baths since the glass sides facilitate reading the capillaries.

e. Reagents. (1) The *index drop solution* has the following formula: 1 part "Aquet" (detergent of Emil Greiner Co.), 500 parts distilled water, a few drops of dilute H_2SO_4 to prevent carbon dioxide absorption by the index drop, and a few crystals of acid fuchsin to give the solution a red color. This fluid flows easily, keeps almost indefinitely at room temperature, and forms an index drop which responds regularly to slight pressure changes. (2) The *carbon dioxide absorbant* is carbonate-free 1 N KOH. (3) The grease used on the ground-glass joints connecting the respiration chambers to the capillaries and on the gaskets of the brass endplates is Dow-Corning silicone stopcock grease. Conventional organic greases

[4] The assistance of Dr. Conrad Yocum in the design of the final capillary volumeter is gratefully acknowledged.

have a tendency to oxidize or react in other ways with oxygen and carbon monoxide under pressure.

Procedure

A roll of filter paper is deposited in the bottom of each respiration chamber and moistened with 0.5 cc. of 1 N KOH. A small paraffin-coated tube is placed in the chamber to support the experimental animal. The latter is inserted and the ground-glass joint in the respiration chamber plugged with the capillary tube.

Four animal-containing respiration chambers, two reference volumeters not containing animals, and three capillary barometers are mounted in a plywood frame (Fig. 1) and held in place with rubber bands. The assembly is then placed in a horizontal position in the compensation chamber with the base of the frame flush against the brass endplate A. By means of a hypodermic syringe 0.05 cc. of the index drop solution is introduced into each of the index drop reservoirs, care being taken not to occlude the capillaries themselves. With valves A and B open, brass endplate B is now bolted on. One then records the temperature of the room, the position of the index drop in the capillary barometers, and the atmospheric barometric pressure.

The experimental gas is supplied from standard cylinders through a manually controlled reduction valve connected by a flexible 0.25″ bore copper tubing to valve A. About 10 liters of experimental gas are flushed slowly through the compensation chamber.[5] Valve B is closed and the experimental gas introduced under pressure through valve A to approximately the desired pressure, as indicated by the capillary barometers. Valve A is then closed.

The compensation chamber is now tilted to a vertical position so that the index drops flow into the lumina of the calibrated capillaries. Valve A is opened slightly and sufficient gas introduced under pressure to force a drop of index fluid a few centimeters into each of the six capillary tubes. Valve A is then closed and the compensation chamber returned to a horizontal position. Valve A is reopened and gas under pressure is slowly admitted until the drops have traversed the lengths of the capillary tubes to the proximal end of the calibrations. Valve A is then closed, the flexible coupling disconnected, and the compensation chamber immersed in a water bath controlled to $\pm$ 0.01° C.

Valve A is now opened carefully until a barely detectable outward movement of the index drops is observed. The rate of gas escape is adjusted so that 10 to 20 minutes are required for the drops to migrate to the distal end of the capillaries. Valve A is finally closed when the distal ends of the index drops are about 2 centimeters from the distal ends of the calibrations. By running the drops up and down in this manner, one wets the walls of the measuring capillaries and thereby assures both a sensitive response and a constant size in the index drops.

Temperature equilibration requires 80 to 100 minutes. After equilibration, the

[5] If this flushing procedure is not carried out, then, upon compression of the compensation chamber with the experimental gas mixture, some of the air in the compensation chamber will be forced into the respiration chambers along with the experimental gas. This introduces considerable error; for example, if the air-filled compensation chamber is compressed with 5 atmospheres of carbon monoxide without prior flushing, the carbon monoxide/oxygen ratio in the compensation chamber will be approximately 25 : 1 while the carbon monoxide/oxygen ratio in the volumeters will be less than 6 : 1.

bath's temperature and the position of the drops in the capillary barometers are recorded to determine the absolute pressure in the compensation chamber. The two barometric pressures that agree most closely are averaged. Readings of the position of the index drop in each calibrated capillary also begin at this time. The positions of the drops are recorded at intervals ranging from 30 minutes to 6 hours, as dictated by the rate of oxygen consumption and the duration of the experiment. Thermobarometric corrections are applied to each reading, taking into account the fact that the actual volume of gas in the reference volumeters is slightly larger than that in the animal-containing chambers. Calculation of thermobarometric corrections may be simplified by enclosing in each thermobarometer a glass rod of approximately the same volume as the animal in the experimental chamber.

To calculate the oxygen consumption in mm.^{3} at S. T. P. from the excursion of the index drop, the following calculations are employed:

Let: v = *volume* in mm.^{3} of capillary that the index drop traversed.
P = absolute pressure in atmospheres after compression.
V_r = gas volume of respiration chamber in cc. (*i.e.*, 45 cc. minus volume of organism and reagents).
V_c = gas volume of compensation chamber in cc. (*i.e.*, 3460 cc. minus volume of 6 respiration chambers and frame = ca. 3000 cc.).
T = Temperature of bath.

A calibration factor F is calculated at each pressure and temperature to convert the measurements of v to mm.^{3} oxygen. Thus:

$$Fv = \text{mm.}^3 \text{ oxygen consumed at S. T. P.}$$

Fenn (1935) has shown that the value of F is provided by the formula

$$F = \left[\frac{V_c + V_r}{V_c}\right] \times P \times \frac{273}{T}.$$

Under ordinary experimental conditions the quantity in brackets is very nearly 1.01. Thus

$$F = P \times \frac{273}{T} \times 1.01$$

$$\text{mm.}^3 \text{ oxygen consumed} = Fv = P \times \frac{276}{T} \times v.$$

Corrections for the vapor pressure of water and for the solubility of oxygen in the insect and the reagents were not applied since the combined errors thereby introduced were less than one per cent.

At the conclusion of the experiment, the compensation chamber is slowly decompressed and unbolted and the animals removed from the respiration chambers. By the addition of acid the total carbon dioxide produced in each respiration chamber during the experiment is displaced from the alkali and measured volumetrically in the gas analyzer described by Bliss (1953) or manometrically by coupling the respiration chamber to a standard Warburg manometer. The average carbon dioxide output may then be calculated.

Accuracy of Method and Range of Application

The calibrated capillaries can be read to ± 0.1 division. This and the total capillary excursion (110 divisions) establish the theoretical limits of accuracy. The actual limits are, of course, determined in large measure by the degree of agreement between the thermobarometers. Table I records the results of a series of readings on two sets of thermobarometers in two typical sets of experimental conditions. The maximum standard deviation of ± 0.16 corresponds to an error of about 0.1 division in reading the positions of the index drops, in close agreement with the theoretical limits. Therefore any individual reading corrected for thermobarometric change is accurate to within ± 0.2 division. A capillary excursion of 30 divisions would thus be accurate to ± one per cent.

Table I

Typical series of thermobarometric readings

Time reading taken (hours)	Difference between initial and subsequent thermobarometric readings					
	T_1	T_2	T_3	T_4	T_5	Standard deviation
	Series 1					
	Five thermobarometers each containing 1 ml. 1 N KOH compressed with 5 atmospheres of nitrogen					
2.00	.8	.8	.8	.9	.7	.07
4.00	1.4	1.4	1.4	1.6	1.4	.08
4.70	1.7	1.8	1.6	1.7	1.6	.07
16.90	2.6	2.8	2.6	2.7	2.4	.16
18.65	2.9	3.0	2.9	3.0	2.8	.07
	Series 2					
	Two thermobarometers each containing 1 ml. 1 N KOH compressed with 5 atmospheres of carbon monoxide					
1.02	.7	.7				0
2.12	1.1	1.3				.15
7.50	2.2	2.3				.1
20.75	1.7	1.5				.15

The experimental method has been applied without difficulty to studies of organisms and tissues having oxygen uptakes between 20 and 1000 mm.^{3}/ hour, and by the use of high pressures of carbon monoxide the role of cytochrome oxidase has been studied in both animals (Schneiderman and Williams, 1954), and plants (Hackett, Yocum and Thimann, personal communication).

We wish to express our sincere appreciation to Professor Carroll M. Williams in whose laboratory these experiments were performed.

Summary

A simple and practical apparatus is described for the measurement of oxygen consumption and carbon dioxide production at positive pressures up to seven atmospheres. It consists, essentially, of a series of capillary respirometers enclosed in a large Lucite compensation chamber capable of withstanding a positive pressure. The details of the construction and operation of the apparatus and the accuracy and range of application are considered.

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THE PHYSIOLOGY OF INSECT DIAPAUSE. IX. THE CYTOCHROME OXIDASE SYSTEM IN RELATION TO THE DIAPAUSE AND DEVELOPMENT OF THE CECROPIA SILKWORM [1]

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During the pupal diapause the respiratory metabolism of the Cecropia silkworm proceeds at a low and relatively constant rate which, except in the case of the intersegmental muscles of the abdomen, is insensitive to carbon monoxide and cyanide. However, with the termination of diapause and the initiation of adult development, a carbon monoxide- and cyanide-sensitive respiration appears and increases progressively, being superimposed on the carbon monoxide-stable respiration of diapause. It was concluded from these and other observations that the metabolism of the developing insect is largely mediated by the cytochrome oxidase system while that of the diapausing pupa is not (Schneiderman and Williams, 1954).

But respiratory measurements in themselves can provide only circumstantial evidence that the coupling of metabolism to cytochrome function is causally related to the termination of diapause and the development which follows. The problem is basically morphogenetic in character and therefore demands solution in morphological terms. Is the change in terminal oxidase coincidental, or is there an obligatory coupling between the function of the cytochrome oxidase system and the actual development of the insect? The present study was designed to answer this question by direct observations of the effects of carbon monoxide on the growth of the Cecropia silkworm during successive stages of metamorphosis.

Materials and Methods

1. Experimental animals

Experiments were performed on embryos, mature larvae, pupae, developing adults, and adults of the giant silkworm *Platysamia cecropia*. The pupae were of three types: (a) Normal diapausing pupae removed from their cocoons and stored continuously at 25° C. ("unchilled diapausing pupae"). (b) Diapausing pupae such as the preceding, except that the brains had been removed and plastic windows established in the facial region and at the tip of the abdomen ("brainless diapausing pupae"). (c) "Previously chilled diapausing pupae"—animals that had been stored at 5° C. for approximately six months and provided with plastic terminal abdominal windows. As previously reported (Williams, 1946), prolonged exposure to low

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temperature renders the brain competent to secrete its hormone and results in the initiation of adult development approximately two weeks after such pupae are returned to 25° C.

In previously chilled pupae provided with plastic windows, the heart-beat and the initiation and day-to-day progress of adult development could be observed directly under the dissecting microscope. As has been emphasized in the previous papers of this series, the visible initiation of adult development is an event of special significance since it signals the end of the months of pupal diapause. Table I records the time sequence of adult development as observed beneath facial and terminal abdominal windows at 25° C., from the first visible signs of hypodermal retraction to the emergence of the adult moth approximately 22 days later. The table records the average tempo of development of a large series and permits one to estimate the stage of development to within ± 12 hours in the vast majority of individuals.

TABLE I

Time-table for the development of male chilled Cecropia at 25° C. as witnessed in pupae equipped with facial and abdominal windows *

Day	*Characters*
0	Initiation of hypodermal retraction just ventral to imaginal disc of genitalia; no retraction elsewhere.
1	Hypodermal retraction under terminal window extends half way up each side; the aedeagus and harpal lobes have tripled in size and migrated slightly toward center of window; hypodermal retraction under facial window has occurred only along posterior margin and is restricted to the midline and the lateral angles; *no retraction of leg hypodermis.*
2	*Initiation of retraction of leg hypodermis,* harpes show considerable enlargement and sharply defined outer edges; beginning of midventral fold between harpes; the aedeagus has migrated about half way to center of window.
3	Facial retraction nearly complete; eye lobes partially visible; terminal retraction complete except dorsally; mid-ventral fold of genitalia extends dorsally to aedeagus; harpes show considerable molding and beginning of subdivision into upper and lower lobes; tips of dorsal harpal lobe slightly forked.
4	Facial and terminal retraction complete; eye lobes well developed but unpigmented; further subdivision of harpes into dorsal and ventral lobes; aedeagus has a cone-shaped, transparent, undivided, membranous tip.
5	Palps and "stalks" of antennae visible for first time. Harpes considerably enlarged and show well developed upper and lower fleshy, semi-transparent lobes; no pubescence; no eye pigment.
6	Membranous tip of aedeagus subdivided into two or three semi-transparent processes; harpal lobes with sharp edges; extremely delicate transparent pubescence along outer edge of upper harpal lobes; no pubescence of lower lobes; no eye pigment.
7	Initiation of pink eye pigment; transparent pubescence now extends along outer edge of lower harpal lobes; genitalia deeply telescoped into preceding segment.
8	Generalized reddish brown eye pigment; genitialia fully formed but fleshy and unpigmented; pubescence generally distributed over outer side of all harpal lobes, but longer and "silky" along edge of upper lobes.
9	Dark reddish brown eye pigment; long silky hairs on upper harpal lobes and shorter silky hairs on lower lobes.
10	Dark brown eye pigment; long silky hairs on all harpal lobes; membranous tip of aedeagus with fleshy spine.
11	No further change.

(Continued on next page)

* The same time-table may also be used for the female insect, save for the characteristics pertaining to the male genitalia. Characters printed in italics are visible without windows and can be seen by moistening the overlying cuticle with 70 per cent alcohol. The adult genitalia of Cecropia have been described and figured by Michener (1952).

TABLE I—*Continued*

Day	*Characters*
12	Tan streak of pigment present on each side of mouth opening; white hairs on upper harpal lobes and on face; earliest tan pigment on genitalia along surface of gnathos and on ridge connecting upper and lower harpal lobes on each side.
13	*Tarsal claws black;* facial cuticle with pale diffuse tan pigmentation; coarse white hairs on harpes; tannish pigmentation of triangular plate (annulus) below base of aedeagus, the pigment extending bilaterally to lower tip of lower harpal lobes; the latter, in turn, show minute black punctate spots; tip of aedeagus dark brown; tan pigmentation of upper harpal lobes; spine on membranous tip of aedeagus still transparent.
14	Spine on tip of aedeagus black; *black, fully-formed antennal barbs.*
15	
16	Persistence of coarse white hairs.
17	*Three black spots along posterior edge of each forewing;* the coarse white hairs on genitalia show initiation of pale pink pigmentation.
18	*Generalized but incomplete wing pigmentation;* red, pink, and white hairs on genitalia; cuticle "soft" only in region of forewings.
19	*Complete wing pigmentation; softening of cuticle extends to dorsum of abdomen.*
20	*Cuticle "soft" throughout but not crisp;* moulting fluid partially absorbed under facial and abdominal windows.
21	*Cuticle crisp throughout; moulting fluid fully resorbed except under abdominal window; cuticle semi-transparent.*
22	*Animal distended; adult emergence.*

2. *Experimental methods*

All experiments were performed at 25° C. Three techniques were utilized in the management of the various gas mixtures:

a. In the *flow method* one or more insects were enclosed in a glass tube through which an approximately streamlined and steady flow of a specific gas mixture was maintained. The mixtures were prepared in pressure cylinders and analyzed prior to use.

b. In the *static pressure method* each animal was placed in a shell vial and the latter loosely plugged with cotton. The vial was then sealed in an individual 2.5-liter air-filled steel chamber and compressed with a specific gas, the pressure being read on a gauge calibrated in pounds per square inch. Alternatively, one or more animals were enclosed in a 3.5-liter air-filled polymethyl methacrylate (Lucite) chamber and compressed with a specific gas. The oxygen tension in the chambers was that of air (20.9 per cent of an atmosphere), while the pressure of the added gas was the gauge pressure. After storage at 25° C. for specific periods the chambers were slowly decompressed, the animals returned to air, and observations continued over a period of several weeks.

c. In the *constant composition pressure method,* a series of insects was placed in a Lucite holder so that their terminal abdominal windows faced uppermost; the holder was then enclosed in a 3.5-liter air-filled Lucite chamber (Fig. 1). The animals were therefore visible through the transparent wall of the chamber and could be studied under the dissecting microscope. A glass trough containing 10 per cent NaOH was placed in the chamber for the purpose of absorbing carbon dioxide. Control experiments revealed that the reaction of carbon monoxide with the concentrated alkali to produce formate occurred so slowly that it did not detectably dimin-

ish the total carbon monoxide pressure. The chamber also contained a calibrated capillary barometer for the measurement of absolute pressure (Schneiderman and Feder, 1954). The air-filled tank was compressed with carbon monoxide, the final pressure being recorded on the tank gauge and the capillary barometer. The latter was read at three-day intervals and the oxygen consumed by the animals replaced by the addition of a corresponding amount of oxygen. On each such occasion a sample of gas was removed and analyzed (Scholander and Roughton, 1943), thus

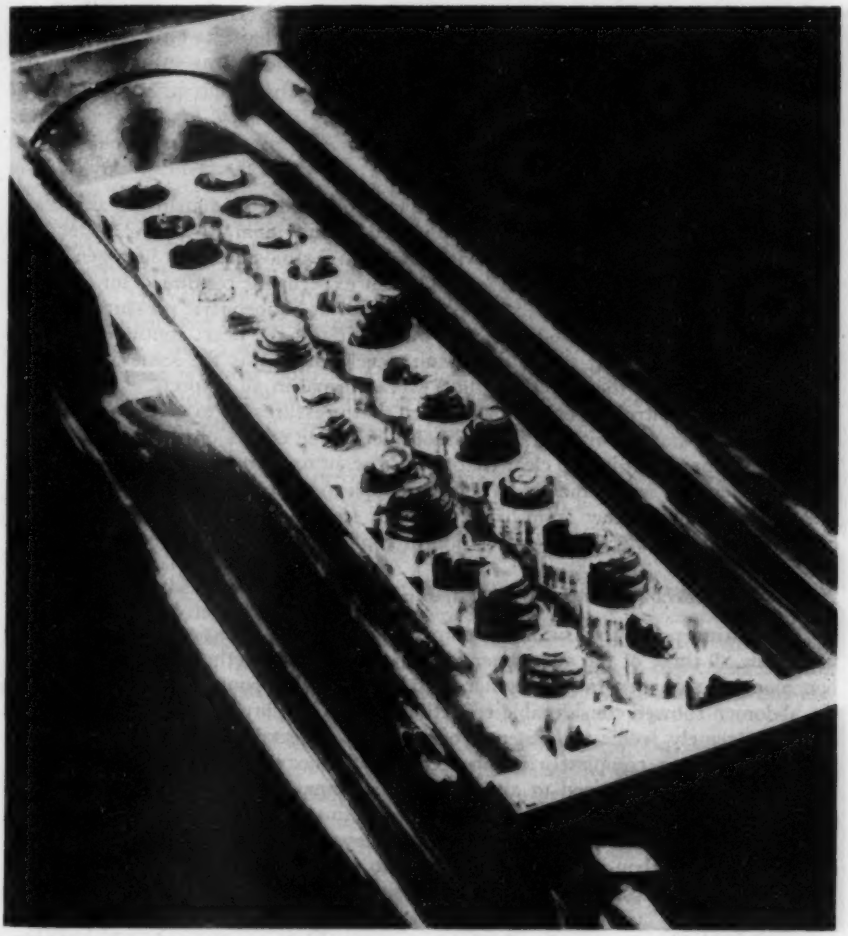

FIGURE 1. Transparent pressure chamber for studying the effects of high pressures of carbon monoxide on the day-to-day progress of development. Thirty animals have been equipped with plastic windows and sealed within the chamber in the presence of five atmospheres of carbon monoxide.

giving double assurance that the oxygen tension in the chamber remained within the desired limits. At the termination of the exposure period the chamber was slowly decompressed, the animals returned to air, and observations continued.

3. Experimental gases

The compressed gases (oxygen, nitrogen, and carbon monoxide) were handled as previously described (Schneiderman and Williams, 1954). In one series of experiments extremely pure carbon monoxide was prepared (*ibid.*). Since the latter was indistinguishable from alkali-washed carbon monoxide in its effects on growth, the less expensive commercially available carbon monoxide was utilized in subsequent experiments.

Results

1. Effects of high pressures of nitrogen

Chilled and unchilled diapausing Cecropia pupae and post-diapausing animals at several stages in adult development were placed in individual air-filled 2.5-liter steel or Lucite chambers and compressed with from 4 to 7 atmospheres of nitrogen. The static pressure technique was utilized and each experiment continued for 21 days. Under this treatment the animals behaved as in air at atmospheric pressure. Spontaneous movements of the abdomen and the beating of the heart continued without interruption. Moreover, the rate of adult development was the same as in air at one atmosphere, and the resulting adults were normal in all respects.

From these control experiments we learn that pressures up to seven atmospheres of an inert gas such as nitrogen are without detectable effects on the adult development of Cecropia. It is also clear that the 525 cc. of oxygen initially present in each air-filled chamber was sufficient to permit a pupa to undergo normal adult development without interference from oxygen lack or from the accumulation of metabolic carbon dioxide during the 21-day period of confinement.

2. Effects of carbon monoxide on diapausing pupae

Diapausing pupae and brainless diapausing pupae were exposed to carbon monoxide by all three of the above-mentioned experimental methods. When the carbon monoxide/oxygen ratio was increased above 10:1, spontaneous movements of the abdomen showed considerable reduction in both amplitude and frequency. Residual extremely feeble movements, occasionally detectable even at 15:1 carbon monoxide/oxygen, completely disappeared after further increase in the ratio. When decompressed and returned to air, normal abdominal motion reappeared within a few hours. In contrast to the paralysis of the intersegmental muscles, the heart continued to beat normally throughout the 21 days of exposure to carbon monoxide even when the carbon monoxide/oxygen ratio was 25:1.

It will be recalled that diapausing pupae initiate adult development after continuous storage at 25° C. for five months or longer (Williams, 1946). This behavior was unimpaired by three weeks of prior exposure to high pressures of carbon monoxide. Evidently, within the diapausing insect the viability of neither the pupal tissues, nor the anlagen of the adult tissues, nor the endocrine organs themselves is dependent on enzymes inhibited by carbon monoxide.

3. Inhibition of wound healing in diapausing pupae by carbon monoxide

Although brainless diapausing pupae are incapable of initiating adult development (Williams, 1946), they retain the ability to repair integumentary wounds. One can study this process to good advantage by removing a disc of hypodermis plus overlying cuticle and covering the wound with a plastic window, the latter being sealed in place with melted paraffin. Spindle-shaped blood cells promptly adhere to the window and begin to string out tenuous cytoplasmic processes. The latter interlace and form a fenestrated tissue which, after 4 or 5 days, is transformed into a transparent, shiny membrane. Meanwhile, the hypodermis begins to close in around the margins of the wound, accompanied by minute tracheae and tracheoles. A continuation of this centripetal growth leads to a central closure of the wound after about 10 to 14 days.

In order to ascertain the effects of carbon monoxide on wound healing, the following experiment was performed. From a series of six previously chilled diapausing pupae the brains were removed and facial and abdominal windows established in each individual. Two days after the operation, three animals were placed in a transparent 3.5-liter air-filled Lucite tank and compressed *via* the static pressure method with five atmospheres of carbon monoxide (carbon monoxide/oxygen ratio of 25:1). The other three animals served as controls and were maintained in air. Each individual was examined daily under the dissecting microscope for signs of regeneration. After a total of 13 days the control group in air had completely repaired the wounds under both facial and abdominal windows. By contrast, the experimental group in carbon monoxide showed no evidence of repair. But when decompressed and returned to air, repair began at once and was completed within 13 days.

Thus, it is clear that even in the diapausing pupa the localized morphogenesis inherent in the repair of a wound is completely inhibited by carbon monoxide.

4. Inhibition of adult development by carbon monoxide

All three of the techniques for the administration of carbon monoxide were utilized in a study of the adult development of previously chilled pupae and of animals that had already initiated adult development. The progress of development in each individual was judged by observations of its genitalia, the day-to-day changes being compared with the normal tempo already defined (Table I). Each experiment was continued for 21 days.

As recorded in Table II it is of special interest that during exposure to carbon monoxide the termination of pupal diapause, as signalled by the onset of adult development, was blocked or greatly delayed. Moreover, individuals which already showed early adult development at the outset of the experiment remained alive in most cases, but further development was either prevented or greatly inhibited.

It is also clear from Table II that the degree of inhibition was a function, not of the carbon monoxide concentration alone, but of the carbon monoxide/oxygen ratio. When the latter was higher than 20:1, development was completely or almost completely blocked. Such animals, when returned to air, promptly resumed normal development where they had left off and produced normal adult moths. However, when development was incompletely blocked in carbon monoxide/oxygen ratios less than 20:1, the insects, upon return to air, continued in a pattern of ab-

TABLE II

Effects of twenty-one days exposure to various carbon monoxide/oxygen ratios on previously chilled Cecropia pupae and on animals at specific stages of adult development

CO/O_2 ratio	Gas content of chamber (atmospheres)	Stage of development at outset	Number of animals	Number of survivors	Average rate of development in CO as % of rate in air	Development after return to air
33:1	6.7 CO+1 air	Previously chilled pupae	6	6	(0.5)	6 normal adults
		<5–25% development	9	8	1	5 normal adults; 3 died
25:1	5 CO+1 air	Previously chilled pupae	8	5	(2)	2 normal adults; 3 died
		<5–25% development	6	5	2	3 normal adults; 2 died
20:1	4 CO+1 air	Previously chilled pupae	8	5	2	No data
19:1	0.95 CO+0.05 O_2	<5–25% development	6	6	11	3 abnormal adults; 1 normal adult; 2 died
		26–50% development	3	3	12	3 slightly abnormal adults
		70% development	1	1	18	1 slightly abnormal adult
15:1	3 CO+1 air	Previously chilled pupae	2	2	(3)	1 abnormal adult; 1 normal adult
		<5–25% development	2	2	15	1 abnormal adult; 1 normal adult
10:1	4 CO+0.2 O_2 +1 air	Previously chilled pupae	8	8	(7)	3 abnormal adults; 3 normal adults; 2 died
		<5–25% development	10	10	14	6 abnormal adults; 4 died
		25–50% development	4	2	25	2 died at 70% stage of development
5:1	1 CO+1 air	Previously chilled pupae	2	2	(15)	No data
1:1	1 CO+0.8 O_2 +1 air	<5–25% development	3	0	70	3 died at 70% stage of development
		Total	78			

Parentheses () indicate that one or more individuals initiated development in the presence of carbon monoxide.

normal development and produced adult moths with various abnormalities. The latter included defective scales, hairs, and pigmentation, along with incomplete or malformed eyes, legs, antennae, and genitalia.

The endocrine competency of the brain itself was found to be unaffected by exposure to carbon monoxide. Thus, brains removed from previously chilled pupae after 21 days of exposure to 25:1 carbon monoxide/oxygen retained their activity and evoked adult development when implanted into brainless diapausing pupae.

5. Effects of carbon monoxide on mature larvae

Mature larvae at the outset of spinning were exposed to carbon monoxide by the static pressure method for one to six days. In control experiments four atmospheres of nitrogen was substituted for the carbon monoxide.

The results recorded in Table III show that neither the behavior nor the viability of the caterpillars was affected by four atmospheres of nitrogen. By contrast, no individual was able to survive exposure to 33:1 carbon monoxide/oxygen for as long as five days. Moreover, in the presence of carbon monoxide/oxygen ratios as low as 1:1, the spinning of a normal cocoon was inhibited, the insect either failing to spin or spinning only a flat sheet of silk.

TABLE III

Effects of various carbon monoxide/oxygen ratios on mature fifth instar Cecropia larvae

CO/O_2 ratio	Gas content of chamber (atmospheres)	Duration of exposure (days)	Type of spinning behavior in chamber	Type of spinning behavior after removal from chamber	Pupated
—	1 air	2	Normal cocoon	Normal cocoon	+
—	4 N_2+1 air	2	Normal cocoon	Normal cocoon	+
33:1	6.7 CO+1 air	5	None	Dead on removal	0
		2	None	Normal cocoon	+
25:1	5 CO+1 air	6	None	Dead on removal	0
20:1	4 CO+1 air	1	None	Normal cocoon	+
15:1	3 CO+1 air	5	Flat sheet	None	+
		4	Flat sheet	Normal cocoon	+
		2	Flat sheet	None	+
		1	Flat sheet	None	+
5:1	1 CO+1 air	3	Flat sheet	Normal cocoon	+
3:1	0.67 CO+1 air	5	Flat sheet	None	+
2:1	0.4 CO+1 air	3	None	Normal cocoon	+
1:1	0.2 CO+1 air	3	None	Normal cocoon	+
		3	Flat sheet	Normal cocoon	+
		3	Flat sheet at first, then continued with a normal cocoon	None	+

6. Effects of carbon monoxide on fertile eggs and embryos

Embryonic development of Cecropia, from oviposition to hatching, requires about ten days at 25° C. From the sixth to the tenth day, one can easily track the progress of embryonic development under the dissecting microscope and thereby estimate the stage of embryonic development.

By means of the static pressure method, freshly oviposited fertile eggs were exposed to a carbon monoxide/oxygen ratio of 20:1 for 1, 3, and 5 days, respectively. Similar experiments were performed on developing embryos which had already completed 10, 30, 70, and 90 per cent of embryological development.

In control experiments compression with four atmospheres of nitrogen was without major effects on viability, and approximately 90 per cent of eggs and embryos hatched. However, even one day of exposure to 20:1 carbon monoxide/oxygen considerably decreased the viability of the embryos. When returned to air, only 10 per cent of the eggs eventually hatched and only 50 per cent showed any detectable progress in embryonic development. Three days of exposure to the 20:1 mixture was lethal in nearly all cases; when returned to air, no eggs hatched and almost all of the embryos were already dead. It is clear that both the development and the viability of eggs and embryos are extremely sensitive to brief exposure to carbon monoxide.

7. Effects of carbon monoxide on the adult moth

By the use of the static pressure method, adult Cecropia moths, 12 to 36 hours after emergence, were exposed to various carbon monoxide/oxygen ratios for periods up to five days. The results summarized in Table IV demonstrate that the

TABLE IV

Effects of various carbon monoxide/oxygen ratios on the viability of adult Cecropia moths

CO/O_2	Gas content of chamber (atmospheres)	Number of animals	Duration of exposure (days)	Behavior in chamber	Behavior after removal from chamber
—	1 air	2	2	Fluttering	Flying
—	4 N_2+1 air	2	2	Fluttering	Flying
33:1	6.7 CO+1 air	2	2	Slight tremors which ceased after 2 hours	Flaccid upon removal. Recovery after 1 and 10 minutes. Feeble coordinated motion within 3 hours, but no flight. Died within 4 days
20:1	4 CO+1 air	2	5	Slight tremors which ceased after 2 hours	Flaccid and dead upon removal
		4	3	Slight tremors which ceased after 2 hours	Flaccid upon removal. Recovery after 10, 10, 20, and 60 minutes. Extremely feeble uncoordinated activity within 3 hours. Died within 3 days without regaining coordination
		2	2	Slight tremors which ceased after 2 hours	Flaccid upon removal. Recovery after 1 and 10 minutes. Considerable coordinated activity within 3 hours, but no flight. Both lived for 6 days after removal, one female laid eggs
		8	1	Slight tremors which ceased after 2 hours	Flaccid upon removal. Recovery after 30 seconds. Flying within 3 hours

moth is definitely sensitive to carbon monoxide. After three days of exposure to a carbon monoxide/oxygen ratio of 20:1, the insects showed considerable decrease in vitality when returned to air; exposure for five days was lethal. Equivalent compression with nitrogen had no effect.

8. Photoreversibility of the carbon monoxide inhibition

Six pupae showing early adult development were placed head-down in an air-filled Lucite tank such as illustrated in Figure 1, and compressed with carbon monoxide to a final carbon monoxide/oxyen ratio of 20:1. Three individuals were illuminated continuously with a 250-watt mercury vapor lamp (General Electric AH-5) *via* their terminal abdominal windows. The light was collected with a reflector and passed through a solution of sodium nitrite to cut off the ultraviolet and through 5 cms. of water to eliminate the infra-red (Bowen, 1949). Three control animals were loosely wrapped in aluminum foil to maintain them in darkness, and

placed in the same chamber. The latter was immersed in a water bath at 25° C., the distance from the light source to the animals being approximately 25 cms.

Exposure to carbon monoxide and simultaneous illumination were continued for 5 days. The chamber was then decompressed and the experimental animals compared with the controls. The genitalia of the illuminated animals had progressed an average of 3.5 days; that is, at 70 per cent of the rate in air. By contrast, the genitalia of the unilluminated individuals showed no detectable progress. This difference was particularly striking at the anterior and posterior ends of the illuminated animals in that the illuminated genitalia showed considerable progress in development whereas the unilluminated facial region showed no morphological advance. Since light-reversibility is a distinguishing property of carbon monoxide's inhibition of cytochrome oxidase, the demonstration of light-reversibility is especially critical, confirming for the insect as a whole the phenomenon as previously encountered in cultures of isolated Cecropia spermatocytes (Schneiderman, Ketchel and Williams, 1953).

9. Effects of oxygen tension on animals at the initiation of adult development

Pupae showing the first day of adult development were exposed to continuously flowing mixtures of oxygen and nitrogen for specific periods, usually 21 days. The results recorded in Table V show that development was retarded by 13 per cent in

TABLE V

Effects of oxygen tension on the adult development of Cecropia (animals on first day of development at outset)

Oxygen tension (per cent of an atmosphere)	Number of animals	Days in gas mixture	Average rate of development as per cent of rate in air	Average rate of development after return to air	Final state
100	3	21	90	2 at 100% normal rate; 1 at 60% normal rate	1 normal adult; 2 slightly abnormal
21 (air)	3	21	100	100%	Normal adults
5	3	21	87	100%	Normal adults
3	3	21	52	100%	Abnormal adults
1	3	15	0	After 10 days in air, development began again and continued at 100% normal rate	1 adult with minimal defects in antennal structure; 2 dead after 45% and 70% development
Less than 0.5	3	7	0	0	Dead when removed from gas

5 per cent of an atmosphere of oxygen, and by 10 per cent in an atmosphere of pure oxygen. Between these limits the rate of development was independent of oxygen tension. Evidently, a gradient in oxygen pressure slightly in excess of 5 per cent of an atmosphere is sufficient to meet the oxygen requirements of the developing tissues. Those individuals which underwent development in the presence of oxygen pressures less than 5 per cent showed abnormalities similar to those encountered after exposure to carbon monoxide (*cf.* section 4).

10. Effects of oxygen tension on mature larvae

Eleven mature larvae were subjected for one to four days to specific low oxygen tensions established by the flow method. The effects were judged in terms of the insect's spinning behavior and subsequent pupation.

Essentially normal cocoons were spun until the oxygen tension was reduced below 3 per cent of an atmosphere. At 2.5 per cent oxygen the animal usually spun silk in a flat sheet (*cf.* section 5). At tensions lower than 2 per cent, spinning ceased; however, animals that had been exposed to this low tension for three days spun normal cocoons when returned to air.

11. Effects of anoxia on larvae, diapausing pupae, and adults

Mature larvae and adult moths were killed by one day of exposure to tank nitrogen containing less than 0.5 per cent oxygen. When diapausing pupae were treated in like manner, the heart ceased to beat after 4 to 7 hours. Half the animals were dead after 72 hours; the survivors, when returned to air, showed resumption of heart beat and abdominal motion after one to two days.

Discussion

1. Systematic changes in sensitivity to carbon monoxide

In the preceding paper of this series, evidence derived from respiratory studies on the Cecropia silkworm demonstrated that marked changes occur in the sensitivity of respiration to carbon monoxide during embryonic and post-embryonic development. The results of the present study reaffirm these changes by demonstrating that diverse physiological activities of the insect show parallel variations in sensitivity to carbon monoxide. In the analysis of these findings it is convenient to subdivide the physiological activities of the insect into processes concerned with "maintenance" and with "growth and activity." The first of these include the minimal metabolic events which sustain the viability and *status quo* of the organism. The second category includes physiological processes responsible for morphogenesis and similar highly involved and specialized activities.

Prolonged survival in the presence of high pressures of carbon monoxide signifies that the gas fails to block the function of any tissue or organ required for the maintenance of life. Death signifies that the function of at least one such tissue or organ is blocked by carbon monoxide. In these terms it is clear that both the maintenance and the growth-and-activity processes are blocked by carbon monoxide in the egg, embryo, and larva. After pupation, however, the maintenance and survival of the diapausing pupa in the dormant state are insensitive to carbon monoxide.

The carbon monoxide-stable mechanism apparently remains intact during the early stages of adult development. But, here also, carbon monoxide continues to block development and to inhibit the contraction of all muscles except the heart. Finally, in the late stages of adult development and in the adult moth, carbon monoxide once again interferes with maintenance as well as with growth and activity.

Evidence has heretofore been presented that the target of carbon monoxide in the insect is cytochrome oxidase (Schneiderman and Williams, 1954). The light-reversibility of carbon monoxide's inhibition of growth is strong confirmation of this

view. Moreover, as was inferred in the previous study, the ability of the diapausing pupa to survive in the presence of high concentrations of carbon monoxide signifies that the loss or inactivation of the carbon monoxide-sensitive cytochrome oxidase system at the time of pupation is compensated by the development of activation of a carbon monoxide-stable respiratory system capable of underwriting the maintenance requirements and the heart-beat of the diapausing insect. This finding affords a remarkably clear illustration in biochemical terms of the dissociability of "maintenance" and "growth" (Needham, 1942, p. 505 ff.).

2. The cytochrome-cytochrome oxidase system and the energetics of development

The dependency of the growth and activity processes of Cecropia at all stages of development upon respiration mediated by cytochrome oxidase finds many parallels. From a study of the literature we have assembled in Table VI a number of processes

TABLE VI

Vital processes in which the inhibitory action of carbon monoxide has been found to be reversed by light

Material		Reference
1. *Arbacia* eggs	Cell division (mitosis)	Clowes and Krahl (1940)
2. Cecropia spermatocytes	*In vitro* spermatogenesis (meiosis and spermiogenesis)	Schneiderman, *et al.* (1951, 1953)
3. *Drosophila*	Adult development	Wolsky (1937)
4. *Avena* (oat)	Growth of isolated coleoptile sections (cell elongation)	Hackett and Schneiderman (1953)
5. *Pisum* (pea)	Growth or isolated stem sections (cell elongation)	Hackett and Schneiderman (1953)
6. *Solanum* (white potato)	Water uptake by tissue slices	Hackett *et al.* (1953)
7. *Daucus* (carrot)	Salt accumulation by tissue slices	Weeks and Robertson (1950)
8. Rat	Incorporation of radioiodine in surviving thyroid tissue	Schachner *et al.* (1943)
9. *Pteridium* (bracken sperm)	Movement of bracken spermatozoids	Rothschild (1951)
10. *Fundulus* (fish) heart	Heart-beat	Fisher and Cameron (1936, 1938)
11. Frog nerve	Action potential	Schmitt (1930)

where a light-reversible carbon monoxide inhibition has been reported. These include meiosis, mitosis, differentiation, cell elongation, water uptake, salt accumulation, flagellar movement, and nerve conduction. As Lemberg and Legge (1949) have reasoned (p. 383): "Whether the respiration of the resting cell is always catalyzed by the cytochrome system or not, it has become increasingly clear that the functional activity of the cell depends on this system." See also Drabkin (1948).

For our present purposes it is of special interest that the inhibition of cytochrome oxidase within the post-diapausing Cecropia establishes and enforces an artificial diapause during the period of exposure of carbon monoxide. It is also noteworthy that even in the diapausing pupa the inhibition of this enzyme prevents wound-healing. From these several lines of evidence we learn that carbon monoxide-sensitive metabolism plays an obligatory role in the energetics of development.

The absence of all but a trace of a complete cytochrome oxidase system in the diapausing pupa therefore assumes special significance (Williams, 1951). Since the presence and function of this system appear to be prerequisite for adult development, its virtual absence in the dormant pupa can, in itself, account for the developmental stand-still of diapause.

In diapausing embryos of the grasshopper, *Melanoplus,* and of the commercial silkworm, *Bombyx,* the absence of a cytochrome-mediated respiration has been attributed to an inactivation of the cytochrome oxidase that is already present; the oxidase is thought to be re-coupled to metabolism in synchrony with the termination of diapause (Bodine and Boell, 1938; Wolsky, 1949). But, in the case of the Cecropia silkworm, the termination of diapause and the onset of development are accompanied by an actual synthesis of a new cytochrome system—not a mere recoupling of enzymes already present (Sanborn and Williams, 1950). The results of the present investigation therefore link the respiratory and enzymatic studies and demonstrate that cytochrome oxidase is the terminal oxidase in processes energizing the insect's development.

The present study confirms the fact that qualitative as well as quantitative changes occur in the energy metabolism of the Cecropia silkworm during the course of metamorphosis. It also contributes to a coherent body of evidence that the cytochrome oxidase system plays an obligatory role in the energetics of morphogenesis. We are therefore persuaded that the recruitment and resynthesis of the cytochrome oxidase system are among the biochemical changes set in motion by the growth and differentiation hormone—changes which couple the endocrine action to the termination of the pupal diapause.

The experiments reported in Sections 5 and 10 were performed in collaboration with Dr. William Van der Kloot and those in Section 9 in collaboration with Mr. Roger Milkman. The photograph in Figure 1 was made by Dr. Roman Vishniac and is used with the permission of *Time,* Inc.

Summary

1. The effects of mixtures of carbon monoxide and oxygen on the growth and metamorphosis of the Cecropia silkworm were examined at successive stages of embryonic and post-embryonic development.

2. Embryos, mature larvae, and adults are killed by five days of exposure to carbon monoxide/oxygen ratios of 20:1 or 25:1. Diapausing pupae, by contrast, survive at least 21 days of exposure to carbon monoxide/oxygen ratios as high as 33:1.

3. While failing to interfere with the viability of diapausing pupae, carbon monoxide blocks or greatly retards the termination of the pupal diapause; it also inhibits the healing of experimental wounds in the pupal integument.

4. The ability to survive in the presence of high pressures of carbon monoxide persists throughout the early stages of adult development. Exposure of the developing, post-diapausing insect to suitable pressures of carbon monoxide establishes and enforces an artificial diapause which is reversed upon return to air.

5. The inhibition of adult development by carbon monoxide is light-reversible; the degree of inhibition is a function of the carbon monoxide/oxygen ratio. These

findings indicate that the effects of carbon monoxide are due to the poisoning of cytochrome oxidase.

6. Resistance to carbon monoxide, as in the diapausing pupa, signals the presence and utilization of an oxidase other than cytochrome oxidase.

7. On the basis of these several lines of evidence, it is concluded that growth and metamorphosis, at all stages in the life history, are dependent on metabolism catalyzed by cytochrome oxidase. The function of cytochrome oxidase is likewise prerequisite for the maintenance of life of the embryo, larva, and adult.

8. Only the diapausing pupa survives without regard to the presence or function of cytochrome oxidase, the maintenance metabolism of the pupae being served by an unidentified oxidase which is insensitive to carbon monoxide.

9. With the termination of pupal diapause the growth and differentiation of the adult moth again requires the function of the cytochrome oxidase system. This fact is considered in relation to the endocrine control of the pupal diapause.

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LETHALITY AND THE BIOLOGICAL EFFECTS OF X-RAYS IN PARAMECIUM: RADIATION RESISTANCE AND ITS VARIABILITY

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It has been known for a long time that *Paramecium* and certain other Protozoa are able to survive exceedingly high dosages of x-rays (see review, Wichterman, 1953). With low, sub-lethal dosages, paramecia become perceptibly accelerated. In normal bacterized culture media, dosages of 200,000 roentgen (r) and above usually retard motility in *Paramecium*, and there are generally no survivors above 510,000 r. Occasionally, survivors of this high dosage produce clones which, after overcoming irradiation effects, reproduce and flourish in a manner comparable to controls (Wichterman, 1948). X-ray survival curves for microorganisms as reported in the literature vary considerably, apparently depending upon the conditions employed for irradiation. We find, for instance, that with certain methods and under certain conditions in the irradiation of *Paramecium caudatum*, the LD 50—that dosage which results in the death of 50 per cent of irradiated organisms—may vary from 75,000 r to 350,000 r.

The purpose of the present investigation was to establish a standard, repeatable method of irradiation and to analyze the causes of radiation resistance and variability in *Paramecium*.

To fully appreciate the insensitivity of paramecia to x-radiation, we need only examine the LD 50 dosages of other organisms. According to Lea (1947), the 50 per cent survival dosage for yeast is 30,000 r; for the bacterium *B. coli*, 5600 r, and for spores of *B. mesentericus*, 150,000 r. For the algae *Chlorella*, *Ankistrodesmus*, and *Chroococcus*, the LD 50 is 22,000 r, 11,000 r, and 9,000 r, respectively (Bonham and Palumbo, 1951). In this connection, it is to be noted that bacteria in culture fluid, as well as those in the body of *Paramecium* and the symbiotic *Chlorella* in *Paramecium bursaria*, can be destroyed by x-rays without killing the paramecia (Wichterman, 1948). It is thus possible to sterilize such cultures to yield species-pure clones of *Paramecium* as well as colorless races of the normally green species, *Paramecium bursaria*. The recent accounts given by Curtis (1951) and Nickson (1952) for some vertebrate animals commonly used in the laboratory are seen to vary, but relatively low dosages of x-rays are required to produce 50 per cent lethality. For instance the LD 50 for "baby" rats is given as 510 r but 590–1280 r for adults. The LD 50 for other animals follows: mice, 400–840 r; guinea

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pigs, 200–310 r; rabbits, 790–1500 r; dogs, 300–335 r; monkeys, 500 r. According to Sparrow and Rubin (1952), it has been estimated that the LD 50 for man would be approximately 400 r when the x-radiation is received over the whole body in a fairly short period of time. It is therefore worthy of note that *Paramecium caudatum*, with an LD 50 of approximately 340,000 r when irradiated in Nylon syringes, has a radiation resistance 850 times as great as that of man and some common vertebrate laboratory animals.

As a test animal for the evaluation of irradiation effects and associated phenomena, *Paramecium* has many useful features. Beginning with a single specimen, it is possible to obtain for experimentation a genetically uniform, pedigreed strain of enormous numbers of paramecia. This allows for speed and precision of observation generally impossible with other test animals. In addition to being a completely isolated cell, *Paramecium* is a structurally complex organism; hence morphologic changes as a result of irradiation can be determined readily. Irradiation effects are manifested in loss of motility, which may include a change in ciliary action or its complete cessation, dysfunction of contractile vacuoles, change in rate of cyclosis, vacuolization, blistering of the pellicle, changes in body shape, and finally disintegration of the body. Also the division rate, which is an index of vitality, can be compared with the control specimens and expressed in quantitative terms. Additional advantages in x-radiation experiments with paramecia may lie in the field of biochemistry, especially in regard to the effects on respiratory mechanisms which appear to be greatly involved.

Materials and Methods

In the present study, all irradiation work was done at the Marine Biological Laboratory, Woods Hole, Massachusetts. The x-ray generator operates simultaneously two water-cooled Coolidge tubes in alternate parallel. One tube was mounted rigidly on a platform on the floor, and the other tube was supported on a counter-balanced arm which allowed it to be moved vertically and in line directly over the fixed tube. Paramecia in irradiation chambers were thus cross-fired from above and below. The x-ray tubes operated at 182 kv. pk., and 25 ma., with an equivalent filtration of 0.2 mm. of copper. When the tubes were brought very close together (position A), which was the position used for all experiments, intensity was 6300 r per minute. Not only were the tubes water-cooled, but an electric fan was directed upon them, and the irradiated materal was surrounded by an ice chamber. Temperature determinations were made by the use of a thermo-junction and galvanometer. The junction was placed directly into the control irradiation chamber; thus it was possible to determine the small temperature changes—which proved to be insignificant—during the entire time specimens were irradiated. Most of the irradiation work was done at a temperature of 15° C.

Although different species of *Paramecium* were irradiated and results indicated species differences in regard to x-ray susceptibility, the results reported here are based upon the use of *P. caudatum*.[3] Cultures were begun with a single specimen and cultivated in covered flasks containing either lettuce or hay infusions which were inoculated with the bacterium *Aerobacter aerogenes* as the food source.

[3] The original strain of *Paramecium caudatum* (57–14) was kindly supplied by Dr. Lauren C. Gilman, University of Miami.

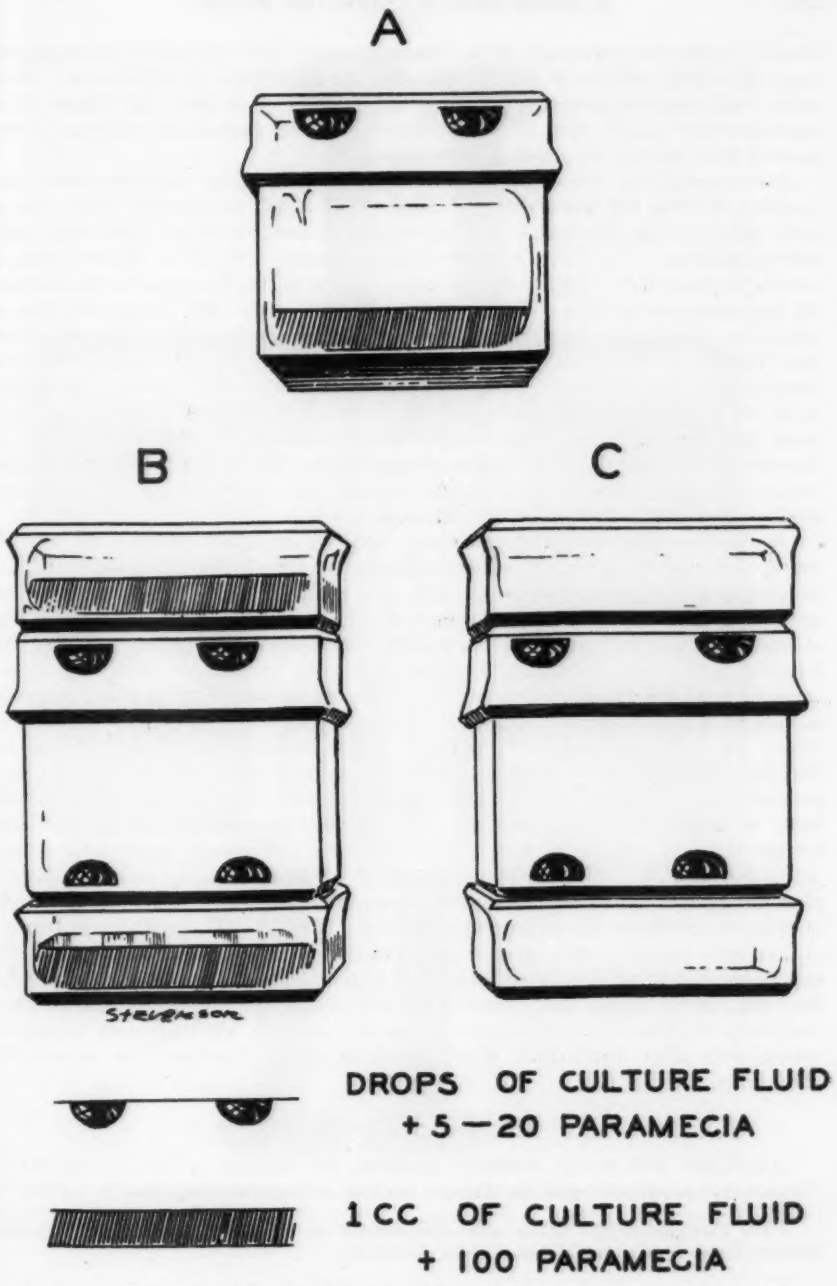

FIGURE 1. Drawings illustrating how conventional plastic boxes were used to irradiate *Paramecium caudatum* in drops and larger volumes of fluid.

Usually vegetative specimens to be irradiated were removed with a micropipette from rich clonal cultures of pH 7.1 following the logarithmic growth phase. Such active and vigorous animals were commonly uniform in size and shape. The environmental culture fluid to be irradiated with the paramecia contained fewer bacteria than during the active growth phase.

For most of the investigations, two types of irradiation chambers were employed. At first, the chambers used consisted of rigid, transparent, plastic boxes with tightly fitting lids and of a type commonly used in such experiments with microorganisms. The boxes measured approximately 24 × 24 × 18 mm. with a volume of about 6 cc. (Fig. 1, A). It was possible to irradiate four boxes containing paramecia at one time. To study the influence of the ratio of the numbers of animals to volume of fluid, drops of uniform size were suspended as hanging drops from the lids of the boxes. The drops, each containing 10, 25, 50 and 100 paramecia, were then irradiated. Additional variations were made utilizing the plastic boxes as shown in Figure 1 and described later. Subsequent experiments indicated that the number of paramecia per unit of volume was not as important in determining the lethal effects of x-radiation as the depth of the exposed culture medium, volume of the moist air-space, and the amount of surface of the culture medium exposed to the air in the radiation chamber.

A new type of radiation chamber was therefore employed to avoid the complicating factor of the air-space which appeared to diffuse from the moist air and which appeared to be extremely lethal to paramecia (Fig. 2). This new chamber consists of a Nylon hypodermic syringe of 2 cc. capacity and graduated in units of one-tenth of a cc. (0.1 cc.). A tightly fitting Lucite cap is applied over the tapering tip of each syringe. The syringe absorbs very little irradiation, eliminates air from the irradiation chamber, and permits the introduction of various substances to be tested during irradiation. The syringes may be sterilized in an autoclave. Accurate sampling of specimens after intervals of irradiation without changing the depth of the medium is also a desirable feature. A Plexiglas holder [4] measuring 11.5 × 8.5 × 2.5 cm. was designed to hold four syringes, all of which could be irradiated at the same time. The syringe-chamber method is thus ideal for the study of lethality of x-rays in *Paramecium* and should prove to be useful for similar studies with other microorganisms. Before sampling and immediately after irradiation, the syringe was quickly rotated between the fingers of both hands in order to distribute the paramecia uniformly. Usually 100 specimens in two cc. of fluid were placed in each syringe and irradiated in steps of 20,000–50,000 r. By expressing 0.2 cc. of irradiated fluid after a given dosage, it was possible to deliver into sterile Pyrex spot plates a precisely countable number of specimens—commonly ten—for the establishment of survival curves. Animals were examined immediately after irradiation, then placed in moist chambers for subsequent observation.

Results and Discussion

Irradiation with x-rays markedly increases the viscosity of the protoplasm of *Paramecium caudatum*; greater dosages lead to irreversible coagulation. Prior to

[4] The Plexiglas syringe holder with self-contained ice chambers was constructed by Mr. Michael Troisi, Instrument Maker, Temple University.

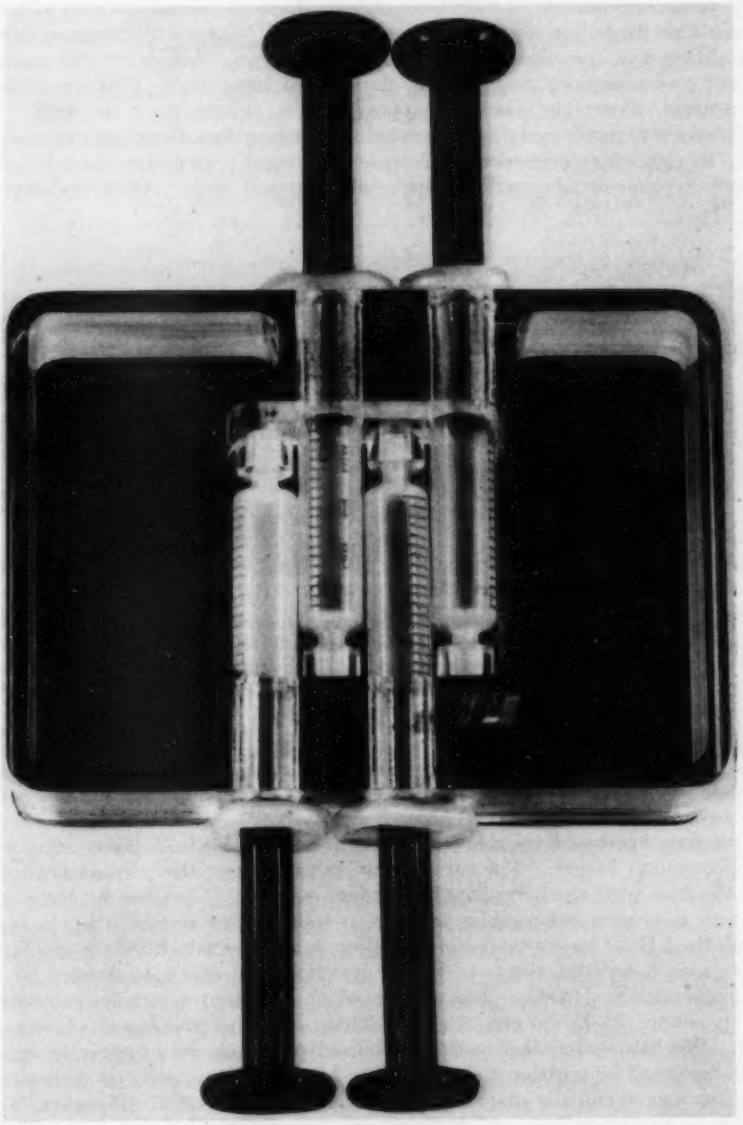

FIGURE 2. Photograph showing four 2-cc. Nylon syringes (with Lucite caps in place) being used as irradiation chambers. An ice well is present on each side of the syringe holder. (Slightly less than actual size.)

death, paramecia become immobilized, change shape to become broadly ellipsoidal and settle on the bottom of the irradiation chamber. Contractile vacuoles function more slowly and sometimes become abnormally large. Active cyclosis ceases as the protoplasm becomes conspicuously darker and vacuolated. Clear, transparent, structureless, blister-like swellings appear on the pellicle prior to death. Near death, waves of trichocysts are extruded, suggesting that these structures—commonly thought of as organelles of defense—represent a response to an injury reaction. Specimens frequently become sub-spherical before their disintegration (Fig. 3).

FIGURE 3. Effects of high dosage x-radiation on *Paramecium caudatum* (× 190). A: Unirradiated control specimen. B: Irradiated with 255,000 r resulting in slight change of body shape; animals generally recover from this dosage. C: Irradiated with 340,000 r (approximately the LD 50 dosage) in which locomotion and cyclosis are retarded. D: Irradiated with 425,000 r in which body shape becomes broadly ellipsoidal; greatly decreased locomotion; vacuolization. E and F: Irradiated with 510,000 r resulting in cessation of locomotion and cyclosis, increased vacuolization, blistering of pellicle, darkening (coagulation) of protoplasm followed by disintegration and death. (Photographs taken of specimens irradiated in Nylon syringes immediately after removal from x-ray generator.)

Our data are based on specimens observed for at least 24 hours after irradiation, commonly longer. The survival curves based upon this method are sigmoid as is the case with most irradiated biological material. Occasionally the slope of the curve is so steep approaching lethality as to be almost vertical. For a 24-hour period, the LD 50 for *Paramecium caudatum* is approximately 340,000 r (Fig. 4). It was soon found that the I. L. D. (immediate lethal dose), as defined by Back and Halberstaedter (1945)—that dosage which produced a complete cessation of motility within 10–15 minutes after irradiation—was not reliable as a useful endpoint. We have found that such immobilized paramecia may appear to be dead, but if examined hours later may be seen to be not only as active as control specimens but may eventually divide and produce successful clones. However, it is of interest to note that Back and Halberstaedter report the I. L. D. to be approximately 350,000 r, a dosage close to our results when using the syringe method.

The results showing percentage survival after irradiating paramecia in drops and larger volumes of fluid in plastic boxes (Fig. 1) and in Nylon syringes are

TABLE I

Survival of Paramecium caudatum after roentgen irradiation in plastic boxes and nylon syringes

Influence of the degree of exposure of animals and culture medium to air during irradiation

Type of chamber	No. of dosage groups	No. of animals observed	Per cent survival dosage in kr.									
			85	128	170	212	255	300	340	383	425	510
Paramecia in hanging drops in 6-cc. plastic boxes containing 1 cc. of culture fluid	4	55			0	0	0	0	0	0		
	12	260			0	0	0	0	0	0		
	12	140			5		0		0			
Paramecia in 1 cc. of culture fluid in bottom of plastic boxes with cover (volume 6 cc.)	10	1000	100									
	4	400			100			0				
	12	335	100		100		0		0		0	0
Paramecia in 1–2 cc. of culture fluid in Nylon syringe (no air bubbles)	36	1335	100	100	95	94	81	57	44	19	2	0

given in Table I. From this tabulation, it may be seen that the paramecia in hanging drops in plastic boxes were much more sensitive to roentgen radiation than the paramecia in the one cc. of culture fluid placed in the bottoms of the plastic boxes (Fig. 1, A). Dosages of 170 kr. killed nearly all of the paramecia in the drops whereas such dosages failed to kill any of the paramecia in the one cc. of fluid in the bottom of the plastic boxes. In most instances, the paramecia placed in hanging drops in the covers of the plastic boxes and the paramecia in the culture fluid in the bottom of the boxes were irradiated simultaneously. Variations in the concentration of paramecia in the drops and in the culture fluid in the bottom of the box did not alter this great difference in radiation sensitivity between drop and culture fluid in the bottom of the box. The only essential difference between these two conditions was the difference in the relative amount of surface exposed to air in the chambers.

It was also quite apparent that even the paramecia in the culture fluid in the bottom of the boxes succumb to the radiation in an almost "all or none" manner. When a dose of 170–200 kr. was exceeded, all paramecia died; in lower dosages, all lived. Some experiments were performed in which the influence of the depth (volume) of the culture medium was tested, since it was thought that variations in culture medium might have been responsible for an x-ray filtration effect. This did not appear to be the reason, however, for the differential sensitivity in drops, as compared with sensitivity in larger volumes of culture fluid. In some experiments, drops with 5–20 paramecia were placed in plastic boxes and one cc. of culture fluid containing 100 paramecia was placed in inverted lids above and below the plastic box chamber containing the drops (Fig. 1, B). The two cc. of culture fluid in the lids thus partially shielded the paramecia in the drops in the boxes. In other similar boxes containing drops with 5 and 20 paramecia per drop, the one

cc. of culture medium above and below was omitted (Fig. 1, C). Both sets of boxes were irradiated with 170 kr. This dose killed all of the paramecia in the drops in both boxes. All of the paramecia in the one cc. of culture fluid in the inverted lids survived. In the case of the paramecia in the inverted lid on top of the box (uncovered and exposed to the atmospheric oxygen at the surface of the culture medium), survival was 100 per cent. Thus it was apparent that all the paramecia in the drops in the plastic container were killed even though they were partially shielded by two cc. of culture fluid (one cc. above and one cc. below).

Because of this differential sensitivity resulting from differences in the degree of exposure of the culture medium to air, the plastic boxes and hanging drops were abandoned and the Nylon syringes were utilized as x-radiation chambers for the reasons given earlier. The irradiation of paramecia in the syringes (which contained no air bubbles) yielded results that were much more uniform. Using the syringe method, the results of seven experiments involving nine different dosage groups and 36 determinations are shown in Table I and the survival curve of Figure 4. In the Nylon syringes, most of the paramecia survived a dose of 170–212 kr. (lethality = 5–6 per cent). As this dosage is exceeded, however, the per cent of animals that survive 24 hours after irradiation takes a sharp drop. Generally no animals survived a 510 kr. dose.

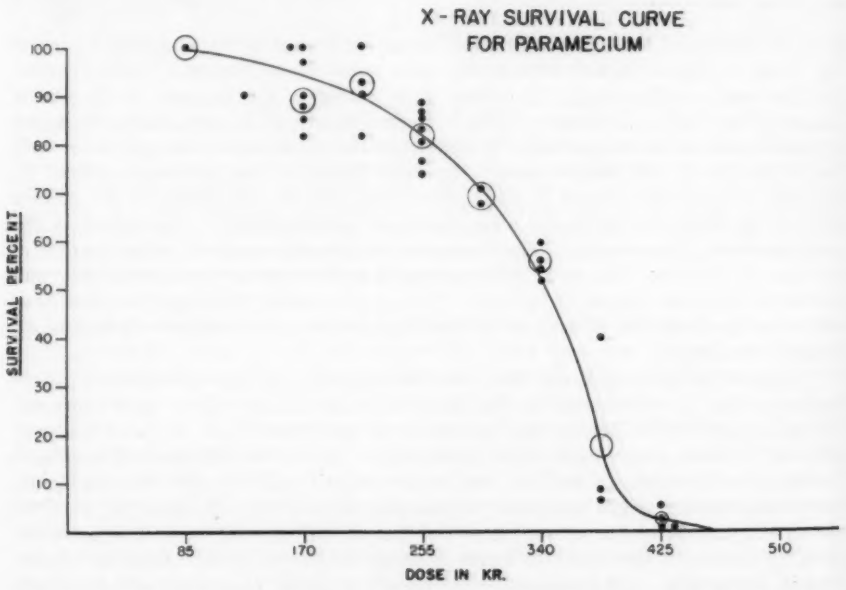

FIGURE 4. X-ray survival curve for *Paramecium caudatum* irradiated in Nylon syringes. This curve is based on seven experiments and after irradiated paramecia were observed for 24-hour period after expulsion from Nylon syringes. Coincident points are not indicated. Each point represents observations on 10–25 counted paramecia.

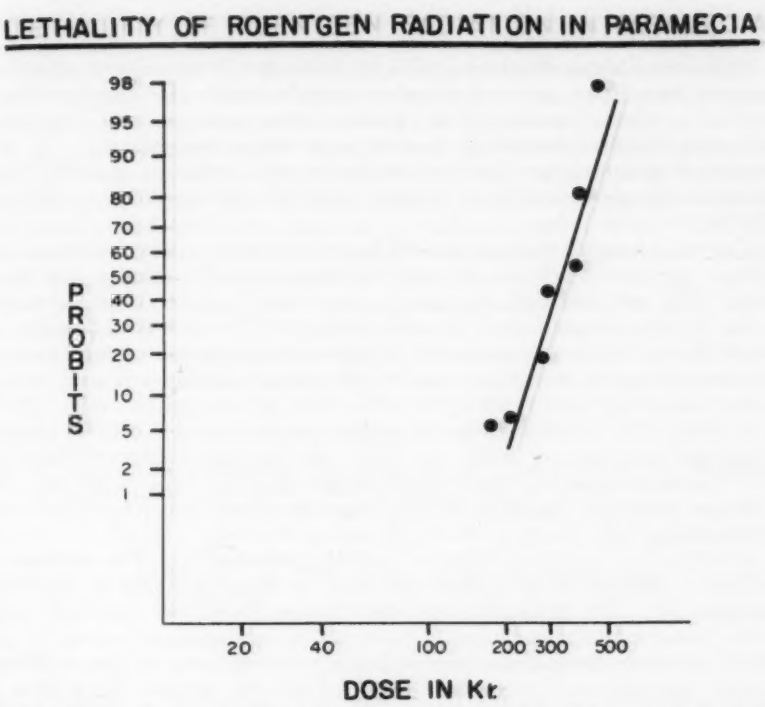

FIGURE 5. Dosage-effect curve for lethality of roentgen radiation in *Paramecium caudatum*. Results were recorded on probability paper for plotting percentages directly on a probit scale.

The data for the per cent survival after irradiation in Nylon syringes were also plotted on log-probit paper (Fig. 5). The significance of such a curve to assist in the analysis of data concerned with all-or-none responses is described by Bliss (1952). From this curve, it may be seen that while there was not a straight line relationship at the higher and lower percentages, one was present in the important range between 10 and 90 per cent. From an examination of this curve, it may be concluded that the LD 50, 24 hours, for *Paramecium caudatum* irradiated in Nylon syringes is approximately 340 kr.

From the experiments with plastic boxes, it was concluded that the number of paramecia per unit of volume was not as important in determining the lethal effects of x-radiation as the depth of the exposed culture medium, volume of the moist air-space, and the amount of surface of the culture medium exposed to the air in the irradiation chamber. This gave rise to the hypothesis that some toxic gaseous substance, possibly ozone (Taylor, 1935) was diffusing into the fluid from the irradiated moist air-space of the chamber. However, we were unable to detect ozone formation in the irradiated air of the chamber, even with the most sensitive tests. The toxic factor derived in whole or in part from the moist air in the closed boxes

during irradiation is probably oxygen or a derivative of oxygen, hydrogen peroxide or some other oxidation product.

When sealed Nylon chambers of air are irradiated with 400 kr. and unirradiated paramecia then drawn into such chambers without outside air being permitted to enter, the paramecia live for as long a period of time as the controls. This shows conclusively that the irradiated air by itself is not toxic to the animals. Also when unirradiated paramecia are placed in irradiated fluid (400 kr.) exposed and not exposed to air, and in irradiated mixtures of air and culture fluid, paramecia are not killed.

It has been known for a long time that water exposed to ionizing radiations forms hydrogen peroxide which may be lethal to ciliates (Taylor, Thomas and Brown, 1933). This does not hold for oxygen-free pure water in which no hydrogen peroxide can be demonstrated even photocolorimetrically (sensitivity 0.1 y per ml.) (Bonét-Maury, 1951). In irradiation chambers containing clear culture fluid with bacterized paramecia, minute amounts of the enzyme catalase originate from the microorganisms and tend to offset the toxic effect of hydrogen peroxide. According to Dale (1951), one molecule of catalase can decompose 5,000,000 molecules of hydrogen peroxide per minute at 0° C. Kimball and Gaither (1952, 1953), using *Paramecium aurelia*, report that hydrogen peroxide is of major importance in the production of certain kinds of nongenetic effects but only under certain circumstances.

A study of the biological effects of ionizing radiations upon *Paramecium* must take into account the effect of these radiations on the environment in which these organisms live. The culture fluid in which the specimens are irradiated consists mainly of water with organic matter from the hay or lettuce infusions. A great body of literature demonstrates that as a result of irradiation of water, hydrogen peroxide, hydrogen and oxygen are formed in which the amounts and relative proportions depend upon such factors as dissolved oxygen concentration, radiation ionic density, dose, temperature and pH. Water that is irradiated oxidizes reducing agents and reduces oxidizing agents (Bonét-Maury, 1951).

In the irradiation of paramecia, another factor that plays a part besides the effect of ionizing radiations of water on the cell is the effect of the accompanying x-rayed bacteria present in the culture as the food source. Experiments in which the irradiated bacteria of paramecia cultures were plated out at intervals up to 350 kr. show the bacteria to have a far lower LD 50 than the paramecia. Another factor to take into account is the indirect or direct effect of radiations of the dead bacteria and their fragmented cells upon paramecia. The experiments with bacteria also showed the necessity of bacterizing spot plates containing irradiated paramecia and fluid if one is to make observations over long periods of time. Failure to do this will result in slower division rates; perhaps ultimate starvation of the paramecia in irradiated paramecia samples.

Summary

1. Irradiation with x-rays markedly increases the viscosity of the protoplasm of *Paramecium caudatum*; greater dosages lead to irreversible coagulation. With increased irradiation, paramecia become immobilized, become broadly ellipsoidal and settle on the bottom of the irradiation chambers. Contractile vacuoles function

more slowly and occasionally become abnormally large. Prior to death, cyclosis ceases and the protoplasm becomes darker and vacuolated. Clear, blister-like swellings appear at the pellicle. Before death, waves of trichocysts are extruded suggesting that their function may represent an injury-reaction. Finally, paramecia frequently become sub-spherical before their disintegration.

2. It was found that one of the most important factors influencing the lethal effects of x-radiation was the degree and extent of exposure of the fluid containing paramecia to air. Paramecia in hanging drops were killed by dosages (170 kr.) that exhibited no lethality for paramecia in larger volumes of culture fluid. This difference in lethality occurred even though the numbers of paramecia per unit volume were kept uniform in both drops and larger volumes.

3. A new method using Nylon syringes was devised to minimize the variability of x-radiation effects.

4. Survival curves were established for *Paramecium caudatum* using this new method. It was found that the LD 50, 24 hours was approximately 340 kr.

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ERRATUM

In the paper by John R. Gregg and Norma Ornstein on "Explant systems and the reactions of gastrulating amphibians to metabolic poisons," which appeared in the December, 1953 issue of THE BIOLOGICAL BULLETIN (Volume 105, No. 3), paragraph (3) on page 476 should read as follows:

"(3) Among the inhibitors that we have studied, sodium barbital is unique in suppressing all three of Em (en, en), St (m, en) and Sp (ec, en), but in allowing Fu (m, ec, en) to occur to some extent. The precise embryological interest of this result is not clear."

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